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TRYPTOPHAN SYNTHETASE ACTIVITY IN PEA SEEDLING EXTRACTS[1, 2]

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Extracts of several microorganisms have been shown to possess tryptophan synthetase activity (2, 5, 6, 8, 9), i.e., such preparations, when incubated in buffer in the presence of indole, serine, and pyridoxal phosphate, catalyze a condensation of indole and serine with the concomitant production of an equivalent amount of tryptophan. Animals, which have a dietary requirement for tryptophan, appear to lack the enzyme, while the one study conducted with higher plant materials has yielded somewhat equivocal results (3). In this work, we present evidence for the existence of tryptophan synthetase activity in pea seedling extracts, and describe various characteristics of the enzyme system.

METHODS AND MATERIALS

PLANT MATERIAL: The plant material most frequently used in these experiments was the seedling of the Alaska pea, the seeds of which were obtained from Asgrow seed company, New Haven, Connecticut. Seeds were soaked in tap water for several hours until the wrinkles disappeared, and were then planted in moist vermiculite in plastic containers. Etiolated seedlings were grown in a dark room, with occasional dim green light as the only illumination, at 27° ± 0.5° C and 50 % relative humidity. Etiolated plants were usually used 7 days after planting. Green seedlings were grown in a controlled room maintained at about 23° C, under mixed warm white and cool white fluorescent lighting of about 1200 ft-c intensity, with either 12 or 16 hours of light per day. These seedlings were usually used 6 days after planting. At this stage the leaflets are included in a compact bud which is much larger than the bud formed on etiolated seedlings.

PREPARATION OF PLANT EXTRACTS: Terminal buds, consisting mainly of unexpanded leaflets, were harvested and placed in a beaker surrounded by crushed ice. The buds were ground with washed, ignited sand and a small volume of phosphate buffer, usually near pH 7, with a mortar and pestle that had been precooled at −15° C. The ground material was then centrifuged near 0° C at 24,000 × G for 30 to 60 minutes. The supernatant fraction was used as the enzyme source. Extracts from other parts of the pea seedling and from other plants were prepared in a similar manner.

CONDITIONS FOR DETECTING TRYPTOPHAN SYNTHETASE ACTIVITY: The general procedure, adapted from the technique used with Neurospora (9) was as follows: To 13 × 100 mm test tubes, were added: 60 micromoles (μM) DL serine in 0.3 ml; 0.2 μM indole in 0.04 ml; 80 μM potassium phosphate buffer in 0.16 ml, usually pH 6.8 or 8.5; and 10 μg pyridoxal phosphate · H_2O in 0.1 ml. The extract to be tested (up to 0.4 ml) and/or distilled H_2O were then added to make a total volume of 1 ml. After incubation in a 30° C water bath for a given length of time (usually 100 minutes), the reaction was stopped by the addition of 0.2 ml 5 % NaOH to each tube. Usually several different volumes of extract were tested in each experiment and controls were included in which serine was omitted, in which enzyme was omitted, and in which NaOH was added before the start of the incubation.

To assay for residual unreacted indole, 3 ml toluene were added to each tube. The tubes were then shaken and centrifuged at low speed for about 1 minute

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[2] This material is part of a dissertation submitted by J. B. Greenberg to the Graduate School of Yale University in June 1958, in partial fulfillment of the requirements for a Ph.D. degree. This work was supported by a grant from the U.S. Public Health Service. Some of the material in this paper was presented at the August 1956 meetings of the American Society of Plant Physiologists at Storrs, Connecticut. An abstract appears in Plant Physiology 31 (Supplement) xxvi, 1956.

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to separate the phases more cleanly. One ml of the toluene layer was removed to a clean test tube, to which 4 ml 95 % ethanol and 2 ml Ehrlich's reagent (9 g *p*-dimethylaminobenzaldehyde plus 45 ml concentrated HCl, made up to 250 ml with 95 % ethanol) were added in that order, and the tubes inverted several times to mix. After 30 to 60 minutes, the color intensity was read on a Klett-Summerson photoelectric colorimeter equipped with the green no. 54 filter. One ml of pure toluene to which ethanol and Ehrlich's reagent had been added served as the blank. The standard curve, at least up to 0.2 μM indole, is very close to linear. Indole disappearance was calculated by comparing the colorimeter readings with controls incubated in the absence of plant extract.

For experiments in which it was desired to subject the product of the reaction to paper chromatography, the reaction was run using 10 times the amount of each constituent in a volume of 10 ml. A no-serine control was included, and also a tube without serine but containing L-tryptophan (1 μM). After incubation for 2 hours at 30° C, the contents of each tube were dialyzed separately against three 10-ml changes of distilled water. The 30 ml of dialysate from each tube was concentrated and reduced to the dry condition under reduced pressure at temperatures below 30° C, and the residues then dissolved in small volumes (about 0.5 ml) of 90 % methanol for application to Whatman 3MM chromatography paper. The developing solvent (isopropanol : ammonia : water, 80 : 5 : 15) was allowed to ascend about 30 cm beyond the line of application of the spots. The sheets were then dried in a fume hood, and sprayed with Ehrlich's reagent, prepared as described above.

Experimental Results [4]

Dependence of Indole Disappearance on Serine, Pyridoxal Phosphate, and Pea Extract: Table I illustrates indole disappearance in the presence

[4] Many of these experiments were performed before we became aware of the fact that the selected pH of 6.8 yielded far less than optimal activity. We believe this not to invalidate any of the conclusions reached in the paper. For details see the section entitled "Effect of pH".

Table I

Tryptophan Synthetase Activity of Pea Extracts

Expt.	Components of extract		μM indole disappearance *										
			Incubation buffer		Experimental: Complete reaction mixture **				Controls: Minus serine		Controls: Minus pyridoxal phosphate		Zero time †
	Tissue	*Phosphate buffer*	μM	pH	*ml of extract*								
					0.1	0.2	0.3	0.4	0.2	0.4	0.2	0.4	0.4
1	7-day green seedlings, leaves, (7 g)	2 ml, 0.02 M, pH 7.1	80	6.8	.038	.070	.088	.112					.000
2	Same as 1, 3 days in freezer, (9.7 g)	1 ml, 0.02 M, pH 7.1	80	6.8	.020	.042	.068	.080	.000	.000			.000
3	6-day green seedlings, buds, (15 g)	1 ml, 0.1 M, pH 7	80	6.8	.028	.063	.096	.118	.000	.014			.000
4	6-day green seedlings, buds, (18.5 g)	5 ml, 0.1 M, pH 6.9	80	6.8	.028	.070	.104	.131	.008	.012			.000
5	6-day green seedlings, buds, (19 g)	6 ml, 0.1 M, pH 6.9	80	6.8	.034	.050	.078	.100		.009	.013	.025	.000
6	10-day green seedlings, buds, (13 g)	2 ml, 0.1 M, pH 6.9	80	6.8		.053		.096		.010		.024	
7	6-day green seedlings, buds (29 g)	5 ml, 0.1 M, pH 7.1	50	8.5	.125	.179	.194	.197		.068		.144	.012
8	Same extract as 7, but 5 days in freezer	...	50	8.5		.120			.026		.038		.007
9	7-day etiolated seedlings, buds, (17 g)	2 ml, 0.1 M,	80	6.8		.049		.086		.025			
10	8-day etiolated seedlings, buds, (6 g)	2 ml, 0.1 M,	80	6.8	.024	.037		.042		.014		.012	.012
11‡	7-day green seedlings, buds (18 g)	2 ml, 0.1 M pH 7.1	80	8.5		.124		.177	.007	.015			

* Incubation time: 100 minutes.

** The complete reaction mixture contains in one ml volume, 60 μM DL-serine, 10 μg pyridoxal phosphate, 0.2 μM indole, plus phosphate buffer and pea extract as indicated.

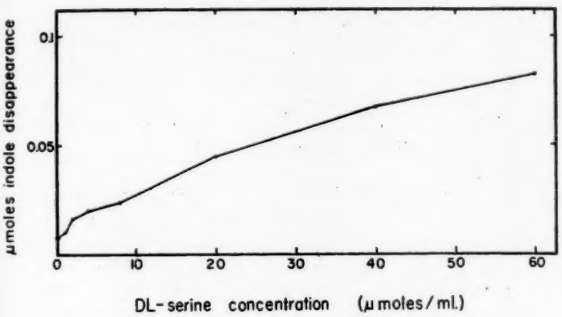
† In "zero time controls," the NaOH was added before incubation instead of after incubation.

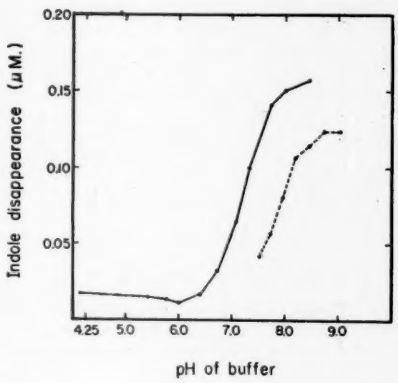
‡ In this experiment the incubation time was 65 minutes, and no NaOH was added.

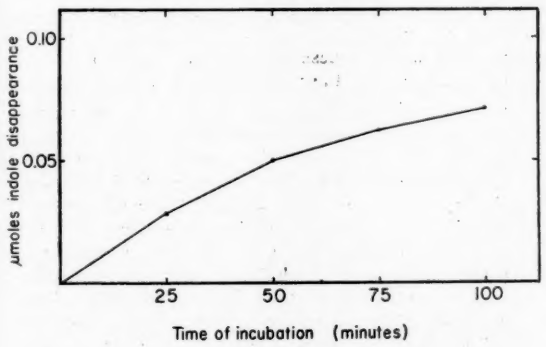
of pea extracts and the extent of its dependence on serine and pyridoxal phosphate. The effect of DL-serine concentration on indole disappearance is illustrated in figure 1. The high molar ratio of serine to indole necessary to bring about significant indole disappearance, is unexplained, but reminiscent of the situation in Neurospora (7) and in *E. coli* (J. Greenberg, unpublished results). The data in table II indicate that 60 μM DL-serine and 30 μM L-serine give about the same activity, suggesting that only the L form of serine participates in this reaction. The experiments in table II also indicate that none of the related compounds tested would substitute for serine in causing indole disappearance in the presence of pea seedling extract.

TIME COURSE OF THE REACTION: The time course of indole disappearance in a complete reaction mixture is shown in figure 2. The extract used here was the same one used in Expt. 3 of table 1, but it had been kept 6 days at −15° C. Each test tube contained 0.4 ml of the extract and 80 μM buffer, pH 6.8. The results indicate a constantly decreasing rate of indole disappearance during the 100 minutes of the experiment.

EFFECT OF pH: The effect of pH on serine-dependent indole disappearance is indicated in figures 3 and 4. The experiments recorded in figure 3 involved plant extracts prepared from buds of 6-day-old light-grown pea seedlings (38 g tissue ground with 5 ml 0.1 M phosphate buffer pH 6.8). Buds of 7-day-old etiolated seedlings (10 g tissue ground with 2.5 ml 0.1 M phosphate buffer pH 7.8) yielded essentially the same results. Three tenths ml of the extract was used for each determination. For the pH-







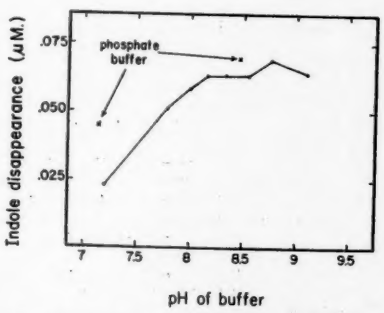


FIG. 1 (*upper left*). The relation between DL-serine concentration and the quantity of indole disappearing from the reaction mixture in 100 minutes.

FIG. 2 (*lower left*). The time course of indole disappearance from the standard reaction mixture containing initially 0.2 μM indole.

FIG. 3 (*upper right*) and FIG. 4 (*lower right*). The effect of pH of the reaction mixture on the rate of indole disappearance. Ordinate refers to μM of indole disappearing. Figure 3. Buds of 6-day-old light-grown seedlings. Solid line, potassium phosphate buffer; dotted line, sodium pyrophosphate buffer-HCl buffer. Figure 4. Extract made from buds of 6-day-old light-grown seedlings. Tris (hydroxymethyl)aminomethane buffer; except for the 2 phosphate buffer points indicated.

activity curves with phosphate buffer, 0.2 ml of 0.5 M phosphate (K^+) at the indicated pH was added to the reaction mixture whose total volume was as usual 1 ml. For the pyrophosphate curves, the buffer was 0.2 ml of 0.125 M sodium pyrophosphate brought to the indicated pH with HCl. The incubation time was 100 minutes.

Figure 4 represents a pH-activity curve in which an extract from buds of light-grown seedlings (29 g tissue ground with 5 ml 0.1 M phosphate buffer pH 7.1) was tested using 0.1 ml of 0.5 M tris (hydroxymethyl)aminomethane buffer adjusted to the indicated pH with HCl. Three tenths ml extract was used for each determination and the incubation time was 75 minutes. Two phosphate buffer data points are included for comparison.

No sharp optimal peaks appear in the pH-activity curves between pH 5 and 9. The pH of each reaction mixture undoubtedly differs to some extent from the pH of the buffer added. However, taken as a whole, the data represented by the several pH-activity curves, involving 3 differently prepared extracts and 3 different types of incubation buffer suggest the following pattern: low activity below pH 7, a sharp increase starting somewhere between 6.5 and 7, and a leveling off somewhere near 8 or 8.5. These pH-activity curves indicate that in the many experiments carried out previously at pH 6.8, the pH was far from optimum. The pH-activity curve reported by Yanofsky (7) for the Neurospora enzyme, using phosphate buffer between pH 7 and 8, indicates an optimum near 7.8.

TABLE II

EFFECT OF COMPOUNDS RELATED TO SERINE ON INDOLE DISAPPEARANCE *

	ML EXTRACT **	ADDENDUM †	μM INDOLE DISAPPEARANCE
Experiment 1	0.1	60 μM DL-serine	0.026
	0.2	" " "	0.051
	0.3	" " "	0.067
	0.4	" " "	0.077
	0.4	30 " "	0.045
	0.4	60 μM L-serine	0.122
	0.4	30 " "	0.080
	0.4	60 μM glycine	0.005
	0.4	30 " "	0.006
	0.4	60 μM DL-threonine	0.006
	0.4	30 " "	0.008
	0.4	60 μM DL-homoserine	0.005
	0.4	30 " "	0.002
	0.4	60 μM DL-alanine	0.002
	0.4	30 " "	0.005
	0.4	60 μM L-cysteine	0.000
	0.4	30 " "	0.005
Experiment 2	0.2	60 μM DL-serine	0.051
	0.4	" " "	0.087
	0.2	60 μM glycolate (K^+)	0.011
	0.4	" " "	0.011
	0.4	none	0.011

* Incubation buffer: 80 μM pH 6.8; Incubation time: 100 minutes; 0.2 μM indole initially present.

** Extract made from buds of 10-day-old light grown seedlings in Experiment 1, and from buds of 6-day-old light grown seedlings in Experiment 2.

† The compounds indicated are used in place of the 60 μM DL-serine usually present.

LABILITY OF THE TRYPTOPHAN SYNTHETASE ACTIVITY OF PEA EXTRACTS: The marked lability of the tryptophan synthetase activity of pea extracts is manifested in several ways. For example, an active extract kept frozen in the deep freeze for 5 days, then thawed and left in the refrigerator overnight showed no activity. The presence of reduced glutathione or of pyridoxal phosphate during the refrigeration did not aid in the retention of activity. These 2 compounds have been found to increase the stability of tryptophan synthetase activity in extracts made from Neurospora (9) and from *E. coli* (J. Greenberg, unpublished).

The rapid decay of activity is further illustrated by the following experiment. An extract causing a 0.131 μM decrease in indole content in a typical reaction mixture was kept for 2 days in the deep freeze, after which time it caused 0.096 μM indole disappearance. The remaining extract was then refrozen and assayed again 2 days later, this time causing only 0.048 μM indole disappearance. Attempts to precipitate the tryptophan synthetase activity with ammonium sulfate were unsuccessful.

ATTEMPTS TO FIND TRYPTOPHAN SYNTHETASE IN OTHER PLANT SOURCES: Thus far, only leaf buds of pea seedlings have been discussed with respect to tryptophan synthetase activity. In other experiments, young expanded leaves were also found to have some activity. Cotyledons yielded a preparation which, in the complete reaction mixture, caused some indole disappearance, but unfortunately controls without serine were not included in these experiments. Other parts of the pea plant tested included stems, root tips, immature pods, and the seeds developing in these pods. These experiments included controls lacking serine, and although in some instances a small amount of indole disappearance occurred, in no case was there any clear enhancement by serine. For example, root tip preparations were found to cause about 0.02 μM indole disappearance under conditions similar to those described in table I, but this occurred both in the presence and absence of serine. The lack of serine dependence could result from a high endogenous serine content relative to tryptophan synthetase content, or from the presence of other indole-utilizing systems. The above experiments were carried out using pH 6.8 or 6.9 phosphate buffer, a factor which may have contributed to the negative or equivocal results.

Several plants other than pea were also tested for activity. Extracts from cabbage seedlings (leaf blades), Black Valentine bean seedlings (buds), radish seedlings (cotyledons and whole seedlings), and tobacco plants (leaves and ovaries) were assayed using pH 6.8 phosphate buffer. Lemna, Spirodela, elm (growing buds) and white lupine seedlings (cotyledons and leaves) were assayed using pH 8.5

phosphate buffer. No clear evidence for the indole-serine reaction could be found in the presence of extracts of any of these plants. Tobacco preparations from both leaves and ovaries were found to induce greater indole disappearance in the absence of serine than in its presence. Experiments with lupine seedlings gave slight indication of tryptophan synthetase activity. However, the results were complicated by the occurrence in lupine cotyledons and leaves of a substance which was extracted from alkaline solutions into toluene, and which gave an orange-red color with Ehrlich's reagent, thus interfering with the indole assay.

CHROMATOGRAPHIC EVIDENCE SUGGESTING TRYPTOPHAN AS THE PRODUCT OF THE REACTION: The serine-dependent indole disappearance in the presence of pea bud extracts would be expected to give rise to tryptophan, by analogy with the reaction known to be catalyzed by microbial preparations. Attempts were made to identify tryptophan as the product of the reaction catalyzed by pea bud extracts. The colorimetric assay with Ehrlich's reagent, as suggested by Nason et al (4) proved unsatisfactory for use with the highly concentrated pea bud extract required in order to get substantial indole disappearance. However, qualitative indications that tryptophan is the product of this reaction come from studies involving paper chromatography. The extracts were prepared as usual from 6-day-old light grown pea seedlings. With isopropanol : ammonia : water (80 : 5 : 15) as the developing solvent, and Ehrlich's reagent as the spray reagent, tryptophan showed up between R_f 0.2 and 0.3, as a purple spot which turned green in time. The R_f was usually depressed somewhat in the presence of the pea bud material. Therefore, controls were run in which tryptophan was spotted both in the presence and absence of pea bud material. Complete reaction mixtures generally yielded a bright purple spot at the R_f of tryptophan, which, in contrast with the minus serine controls, turned green in time, as did pure tryptophan. Controls in which the incubation was carried out in the absence of serine, or in which pea extract was incubated alone, yielded a light purple spot at the R_f of tryptophan, but after some time the spot seemed to fade completely instead of turning to green. Good et al (1) found spots corresponding to tryptophan on paper chromatograms of all 12 different plant species studies, including etiolated pea epicotyls. Our data therefore probably show that the extracts of light-grown pea buds contained some tryptophan, but that additional tryptophan was formed from the condensation of indole with serine.

DISCUSSION

From the results presented in this paper, we may infer the existence in pea seedling buds of an enzyme which, in the presence of pyridoxal phosphate, catalyzes the coupling of indole and serine to form tryptophan. While the results of the paper chromatography experiments reported here strongly suggest that tryptophan is the product of the reaction, a suitable tryptophan assay would be desirable in order to check the quantitative relationship between indole disappearance and tryptophan formation. Bioassay methods using a bacterial mutant capable of growing on tryptophan but not on indole might prove useful in this regard.

In this study it has not been possible to detect significant serine-dependent indole disappearance in extracts prepared from plants other than the pea. It would seem unlikely, however, that tryptophan is made one way in various microorganisms and in the pea plant, and in another way in the remainder of the higher plants. The instability of pea breis with respect to tryptophan synthetase activity, and the requirement for very concentrated extracts to detect significant activity, have already been mentioned. In light of this, further experiments at proper pH values using carefully prepared concentrated extracts of plant tissues actively synthesizing new cellular materials might well show tryptophan synthesis from indole and serine to be the general pattern among higher plants.

One may argue that the presence of tryptophan synthetase activity in pea brei does not prove that in the pea plant tryptophan is made in this way. The problem of the validity of extrapolation from tissue brei to intact plant is difficult to settle. However, the natural occurrence in higher plants of the 2 substrates and the product, as well as the inability of naturally occurring products related to serine to replace serine, make it reasonable to accept this extrapolation tentatively.

Recent investigations (10) suggest that although both Neurospora and *E. coli* yield enzyme preparations that couple indole and serine to form tryptophan, the reaction in vivo in Neurospora probably involves indoleglycerophosphate rather than free indole. This reaction should also be tested for in higher plant preparations.

In a recent publication, Mudd and Zalik (3) reported finding indole disappearance, measured colorimetrically, in the presence of various tissues and cell free extracts prepared from the tomato plant. Qualitative colorimetric assay suggested that tryptophan was a product of the reaction. Serine enhanced a decrease in indole in the presence of cotyledons incubated for 8 hours at room temperature. However, in the presence of cell-free extracts, which were effective in causing indole disappearance, no enhancement by serine could be detected. Thus it is not clear whether in these experiments indole disappearance was in fact due to the coupling of indole with serine.

SUMMARY

The buds of pea seedlings, both light-grown and etiolated, yielded extracts, which when incubated in the presence of indole, serine, pyridoxal phosphate, and buffer, catalyzed the disappearance of indole. In the absence of serine the extent of indole disappearance was substantially reduced, in some cases almost to zero. Increased concentrations of serine

resulted in increased indole disappearance. The omission of pyridoxal phosphate also markedly reduced the extent of indole disappearance.

L-Serine rather than D-serine appeared to be involved in the coupling reaction. Glycine, threonine, homoserine, cysteine, alanine and glycolate were all unable to substitute for serine. pH dependence was studied in 3 different buffer systems. The overall results suggest low activity below pH 7, a sharp increase starting somewhere between 6.5 and 7, and a leveling-off somewhere near 8 or 8.5. Indole disappearance progressed at a constantly decreasing rate during the 100 minute incubation period studied.

Expanded pea leaves also yielded active extracts. Preparations from stems, root tips, pods, and developing seeds, as well as plants other than the pea, did not promote serine-dependent indole disappearance. The results with pea cotyledons were uncertain. The tryptophan synthetase of pea buds appears to be very labile, and it is believed that the observed lack of activity in extracts of other plants may be partly attributable to enzyme lability.

Chromatographic evidence suggests that in the pea bud system, the product of serine-dependent indole disappearance is tryptophan. It is concluded that pea bud extracts contain an enzyme analogous to tryptophan synthetase in microorganisms, and that tryptophan synthesis in the pea plant probably follows the same pathway as in microorganisms.

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IDENTIFICATION OF THE ANTHOCYANINS IN PETALS OF TULIP VARIETIES SMILING QUEEN AND PRIDE OF HAARLEM [1]

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Willstatter and Bolton (9) were the first to identify the pigments from petals of *Tulipa gesneriana*. They found that the scarlet-red color of some varieties was due to a mixture of cyanidin diglucoside (cyanin) and carotenoids. Robinson and Robinson (6) found that garden tulips contained either a mixture of cyanidin and pelargonidin biosides or cyanidin bioside and delphinidin diglucoside. They stated that "the examination of the anthocyanins of the tulip is much more difficult than that of most other plants." Recently the anthocyanin from the tulip variety Queen of the Night was identified by Shibata (7) as delphinidin rhamnoglucoside and Shibata and Sakai (8) identified the anthocyanin of the variety Eclipse as cyanidin rhamnoglucoside. There is a wide range of complexity in the anthocyanin constitution of tulips ranging from the simple one component found by Shibata (7) and Shibata and Sakai (8) to the more complex multiple components found in the varieties examined by Robinson and Robinson (6).

The purpose of the present investigation was to isolate and identify by chromatographic and spectrophotometric methods the anthocyanins from the petals

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of the tulip varieties Smiling Queen and Pride of Haarlem. These varieties were chosen because a preliminary examination had indicated that they contain several pigments.

Methods and Materials

Smiling Queen: Petals were dried at 50° C in a forced-draft oven and then ground in a Wiley mill to pass a 20-mesh screen. The ground tissue was extracted in cold methanol containing 1 % hydrochloric acid by blending for 5 minutes. The extract was filtered and the anthocyanins were precipitated with neutral lead acetate. The supernatant was discarded, and the lead salt was washed with distilled water and then air-dried at room temperature. The anthocyanins were recovered from the lead salt by blending with methanol containing 2 % hydrochloric acid for 5 minutes. The lead chloride precipitate was removed by filtration. Excess lead in the methanolic anthocyanin extract was precipitated by passing hydrogen sulfide through the solution and it was removed by centrifugation. The methanolic anthocyanins solution was then concentrated under reduced pressure at 40° C, and the anthocyanins were precipitated with ether. The anthocyanins precipitate was dissolved in a minimum volume of methanol containing 1 % hydrochloric acid and placed at 4° C for 3 days. In the cold a brown-yellow precipitate formed and was removed by filtration and discarded. The methanolic anthocyanins solution was then taken to dryness under reduced pressure at 4° C and the residue dissolved in a minimum volume of 1 % aqueous hydrochloric acid. The aqueous anthocyanins solution was shaken several times with ethyl acetate to remove yellow flavonoid pigments and the clear red solution containing the anthocyanins was subjected to a paper chromatographic separation.

Chromatograms were prepared by streaking the red solution in a band approximately 1 cm wide across the narrow width of Whatman no. 3 MM paper (18 × 22 inches). The papers were irrigated in a chromatographic cabinet by the descending method in a solvent of 1-butanol : acetic acid : water (6 : 1 : 2 v/v). The anthocyanins separated into 3 bands and each band was cut out and eluted with methanol containing 5 % acetic acid. The separated anthocyanins were rechromatographed, as previously described by using 15 % acetic acid as the solvent. The 1st band separated with the 1-butanol : acetic acid : water (6 : 1 : 2 v/v) solvent resolved into 2 components in 15 % acetic acid. The 4 anthocyanins obtained were further purified by rechromatographing with 1-butanol : acetic acid : water (6 : 1 : 2 v/v) on water-washed Whatman no. 3 MM filter paper.

A 1 ml portion of the purified, concentrated anthocyanin was hydrolyzed with 1 ml of 2 *N* hydrochloric acid by heating at 100° C under reflux for 45 minutes. The anthocyanidin fraction was extracted with a minimum volume of *iso*-amyl alcohol and the aqueous phase was examined for sugars as described by Asen et al (1).

R_f values for the anthocyanins and anthocyanidins were determined by ascending chromatography. A suitable portion of each extract was applied at 1 inch intervals along the starting line on 15 × 16 inch sheets of Whatman no. 1 filter paper rolled into cylinders as described by Irreverre and Martin (5) and irrigated with solvents listed in table I and II. Detection of the anthocyanins and anthocyanidins was

Table I

R_f Values and Wave Lengths of Maximum Absorption for the Anthocyanins of Tulip var. Smiling Queen

Compounds	R_f values in indicated solvent							λ Max in ethanol containing 0.01 *N* HCl (Mμ)
	a	b	c	d	e	f	g	
Tulip anthocyanins:								
Band no. 1	.27	.35	.46	.49	.38	.41	.25	537
Band no. 2	.32	.33	.34	.45	.32	.56	.39	539
Band no. 3	.36	.47	.62	.74	.40	.59	.32	517
Band no. 4	.42	.46	.67	.75	.48	.46	.40	517
Authentic anthocyanins:								
Cyanidin-3-glucoside	.33	.34	.43	.51	.35	.43	.24	538
Cyanidin-3-galoctoside	.32	.32	.51	.61	.32	.44	.25	538
Cyanidin-3-rhamnoglucoside	.29	.32	.35	.47	.34	.56	.35	539
Pelargonidin-3-glucoside	.44	.43	.67	.79	.51	.45	.32	518
Pelargonidin-3-rhamnoglucoside	.30	.45	.65	.77	.43	.57	.41	517
Delphinidin-3-glucoside	.12	.21	.17	.30	.21	.29	.19	552

Solvent:

a. 1-Butanol : acetic acid : water (6 : 1 : 2 v/v)
b. 1-Butanol : 2 *N* hydrochloric acid (1 : 1 v/v)
c. *m*-Cresol : acetic acid : water (50 : 2 : 48 v/v)
d. Phenol : water (73 : 27 w/w)
e. Ethyl acetate : *t*-Butyl alcohol : acetic acid : water (5 : 4 : 1 : 3)
f. Acetic acid (15 %)
g. Acetic acid (5%)

made by co-chromatography with authentic compounds.

The absorption spectra of the anthocyanins and anthocyanidins were determined by using an ethanolic solvent containing 0.01 *N* hydrochloric acid.

PRIDE OF HAARLEM: A 15 g sample of dried ground petals was extracted in 500 ml of methanol containing 1 % hydrochloric acid for 20 hours at room temperature. The extract was filtered and the anthocyanins precipitated with neutral lead acetate. Only a portion of the anthocyanins precipitated as the lead salt and the lead salt precipitate was removed by filtration. A considerable amount of the pigment remained in solution even when excess neutral lead acetate was added. The color of the solution indicated that it contained principally pelargonidin glycosides. Excess lead was precipitated by passing hydrogen sulfide gas through the solution and was removed by centrifugation. The solution was taken to dryness under reduced pressure at 40° C and the residue was dissolved in a minimum volume of methanol. The anthocyanins were precipitated with ether and kept at −5° C for 24 hours. The ether-precipitated anthocyanins were recovered by filtration and dissolved in a minimum volume of methanol. The anthocyanins precipitated by neutral lead acetate were recovered by blending for 5 minutes in methanol containing 2 % hydrochloric acid and filtering. The anthocyanin extracts from the ether precipitate and the lead precipitate were combined and further purified by reprecipitating from ether 4 more times. The anthocyanins from the last ether precipitation were reconstituted in water and extracted 5 times with ethyl acetate to remove other flavonoid pigments. The aqueous anthocyanins solution was taken to dryness under reduced pressure at 40° C and the residue dissolved in a minimum volume of methanol, precipitated with ether and kept at −5° C for 3 days.

The isolation and purification of the anthocyanins were accomplished by column chromatography since Harborne (4) found that arabinose could be produced as an artifact during the purification of anthocyanin on Whatman no. 3 filter paper. Columns of Whatman cellulose powder 40 by 500 mm were prepared for each solvent used to resolve the anthocyanins. The columns were packed in approximately 5 cm bands by pouring a suitable volume of a suspension of the cellulose powder in the solvent into a 10 cm column of the solvent and allowing the cellulose powder to settle completely before pouring the next portion.

The anthocyanin was dissolved in a minimum volume of 1-butanol : acetic acid : water (6 : 1 : 2 v/v), placed on the top of the cellulose powder column and developed with the same solvent. The anthocyanin resolved into 3 bands, which were collected automatically in 5 ml fractions. The resolved bands were contaminated with yellow pigments and compounds which fluoresced in ultra-violet light. The 1st and 2nd band resolved by 1-butanol : acetic acid : water (6 : 1 : 2 v/v) were further purified by re-chromatographing with 15 % acetic acid. In this solvent the 2nd band separated into 2 components. The 3rd band resolved by 1-butanol : acetic acid : water (6 : 1 : 2 v/v) was further purified by developing with 2 *N* hydrochloric acid : 1-butanol (1 : 1 v/v) followed by 15 % acetic acid. In the 15 % acetic acid solvent, the 3rd band separated into 2 components. The resolved 5 pigments were identified by methods previously described.

RESULTS

SMILING QUEEN: The R_f values and the wave length of maximum absorption for the 4 anthocyanins resolved indicated that 2 are cyanidin glycosides (bands 1 and 2) and that 2 are pelargonidin glycosides (bands 3 and 4) (table I). The 2 cyanidin glycosides

TABLE II

R_f VALUES AND WAVE LENGTHS OF MAXIMUM ABSORPTION FOR ANTHOCYANIDINS FROM ACID HYDROLYSIS OF THE ANTHOCYANINS IN TULIP VAR. SMILING QUEEN

COMPOUND	R_f VALUES IN INDICATED SOLVENT			λ MAX IN ETHANOL CONTAINING 0.01 *N* HCl (Mμ)
	a	b	c	
Tulip anthocyanidins:				
Band no. 1	.56	.76	.65	544
Band no. 2	.56	.75	.64	544
Band no. 3	.75	.84	.84	529
Band no. 4	.75	.84	.85	530
Authentic anthocyanidins:				
Cyanidin	.56	.76	.65	545
Pelargonidin	.76	.84	.85	530
Delphinidin	.37	.46	.33	555
Petunidin	.53	.55	.48	557

Solvents:
a. Acetic acid : hydrochloric acid : water (30 : 3 : 10 v/v)
b. 1-Butanol : 2 *N* hydrochloric acid (1 : 1 v/v)
c. *iso*-Amyl alcohol : hydrochloric acid : water (5 : 1 : 1 v/v)

were best separated by *m*-cresol : acetic acid : water (50 : 2 : 48 v/v), 15 % acetic acid and 5 % acetic acid, while the pelargonidin glycosides were best resolved by ethyl acetate : *t*-butyl alcohol : acetic acid : water (5 : 4 : 1 : 3), 15 % acetic acid, and 5 % acetic acid.

To identify the anthocyanins further, the 4 pigments were acid-hydrolyzed and the products were identified by paper chromatographic and spectrophotometric methods. The anthocyanidin produced from bands 1 and 2 was cyanidin and from bands 3 and 4 pelargonidin (table II). Hydrolysis of bands 1 and

TABLE III

R_g VALUES AND COLOR WITH ANILINE HYDROGEN PHTHALATE OF SUGARS FROM ACID HYDROLYSIS OF ANTHOCYANINS IN PETALS OF TULIP VAR. SMILING QUEEN

COMPOUND	R_g VALUES IN INDICATED SOLVENT			COLOR WITH ANILINE HYDROGEN PHTHALATE
	a	b	c	
Sugars from acid hydrolysis of tulip anthocyanins:				
Band no. 1	1.00	1.00	1.00	Brown
Band no. 2	1.01	1.00	1.01	Brown
	1.68	1.62	1.53	Brown
Band no. 3	1.00	0.99	1.00	Brown
	1.69	1.65	1.52	Brown
Band no. 4	1.00	1.00	1.00	Brown
Authentic sugars:				
Glucose	1.00	1.00	1.00	Brown
Galactose	0.91	0.95	1.10	Brown
Lyxose	1.40	1.27	1.28	Red
Arabinose	1.17	1.16	1.33	Red
Xylose	1.39	1.31	1.21	Red
Rhamnose	1.69	1.63	1.53	Brown

Solvents:
a. Ethyl acetate : pyridine : water (8 : 2 : 1 v/v)
b. 1-Butanol : ethanol : water (40 : 11 : 19 v/v)
c. Phenol : water (73 : 27 w/w)

TABLE IV

R_f VALUES AND WAVE LENGTHS OF MAXIMUM ABSORPTION FOR THE ANTHOCYANINS OF TULIP VAR. PRIDE OF HAARLEM

COMPOUND	R_f VALUES IN INDICATED SOLVENT						λ MAX IN ETHANOL CONTAINING 0.01 *N* HCl (Mμ)
	a	b	c	d	e	f	
Tulip anthocyanins:							
Band no. 1	.15	.13	.35	.44	.25	.44	553
Band no. 2	.25	.30	.34	.55	.38	.53	539
Band no. 3	.30	.32	.44	.57	.36	.43	538
Band no. 4	.34	.45	.60	.72	.47	.55	518
Band no. 5	.43	.42	.63	.78	.50	.43	518
Authentic anthocyanins:							
Cyanidin-3-glucoside	.30	.31	.46	.61	.38	.41	538
Cyanidin-3-galoctoside	.28	.30	.48	.70	.36	.43	538
Cyanidin-3-rhamnoglucoside	.27	.33	.34	.56	.40	.53	539
Pelargonidin-3-glucoside	.41	.45	.68	.80	.51	.42	518
Pelargonidin-3-rhamnoglucoside	.35	.42	.60	.73	.50	.55	518
Delphinidin-3-glucoside	.19	.16	.17	.45	.25	.27	552
Delphinidin-3-rhamnoglucoside	.16	.16	.36	.44	.24	.44	552

Solvents:
a. 1-Butanol : acetic acid : water (6 : 1 : 2 v/v)
b. 1-Butanol : 2 *N* hydrochloric acid (1 : 1 v/v)
c. *m*-Cresol : acetic acid : water (50 : 2 : 48 v/v)
d. Phenol : water (73 : 27 w/w)
e. Ethyl acetate : 3-Butanol : acetic acid : water (5 : 4 : 1 : 3 v/v)
f. Acetic acid (15 %)

4 yielded 1 sugar identified as glucose and that of bands 2 and 3, 2 sugars, identified as glucose and rhamnose, (table III). The 4 anthocyanins in the petals of the tulip variety Smiling Queen are cyanidin-3-glucoside (chrysanthemin), cyanidin-3-rhamnoglucoside (antirrhinin), pelargonidin-3-glucoside (callistephin), and pelargonidin-3-rhamnoglucoside.

PRIDE OF HAARLEM: The R_f values and the wave length of maximum absorption for the 5 anthocyanins resolved indicate that there is 1 delphinidin glycoside (band 1), 2 cyanidin glycosides (bands 2 and 3) and 2 pelargonidin glycosides (bands 4 and 5) (table IV).

Acid hydrolysis of the anthocyanins yielded delphinidin from band 1, cyanidin from bands 2 and 3 and pelargonidin from bands 4 and 5 (table V). The sugar moiety of the anthocyanins yielded glucose for bands 3 and 5 and glucose and rhamnose for bands 1, 2, and 4 (table VI). The 5 anthocyanins in the petals of the tulip variety Pride of Haarlem are delphinidin-3-rhamnoglucoside, cyanidin-3-rhamnoglucoside (an-

TABLE V

R_f VALUES AND WAVE LENGTHS OF MAXIMUM ABSORPTION FOR ANTHOCYANIDINS FROM ACID HYDROLYSIS OF THE ANTHOCYANINS IN TULIP VAR. PRIDE OF HAARLEM

COMPOUND	R_f VALUES IN INDICATED SOLVENT			λ MAX IN ETHANOL CONTAINING 0.01 *N* HCl (Mμ)
	a	b	c	
Tulip anthocyanidins:				
Band no. 1	.38	.43	.34	555
Band no. 2	.56	.72	.70	545
Band no. 3	.56	.73	.70	544
Band no. 4	.77	.78	.82	530
Band no. 5	.77	.79	.82	529
Authentic anthocyanidins:				
Cyanidin	.55	.73	.70	545
Pelargonidin	.77	.79	.82	530
Delphinidin	.37	.41	.34	555
Petunidin	.54	.52	.47	557

Solvents:
a. Acetic acid: hydrochloric acid: water (30: 3: 10 v/v)
b. 1-Butanol: 2 *N* hydrochloric acid (1: 1 v/v)
c. *iso*-Amyl alcohol: hydrochloric acid: water (5: 1: 1 v/v)

TABLE VI

R_g VALUES AND COLOR WITH ANILINE HYDROGEN PHTHALATE OF SUGARS FROM ACID HYDROLYSIS OF ANTHOCYANINS IN PETALS OF TULIP VAR. PRIDE OF HAARLEM

COMPOUND	R_g VALUES IN SOLVENT			COLOR WITH ANILINE HYDROGEN PHTHALATE
	a	b	c	
Sugars from acid hydrolysis of tulip anthocyanins:				
Band no. 1	1.00	0.98	1.01	Brown
	1.86	1.56	1.62	Brown
Band no. 2	1.00	0.99	1.00	Brown
	1.92	1.61	1.59	Brown
Band no. 3	1.01	0.98	1.00	Brown
Band no. 4	1.00	1.00	0.98	Brown
	1.94	1.59	1.60	Brown
Band no. 5	1.00	0.98	1.00	Brown
Authentic sugars:				
Glucose	1.00	1.00	1.00	Brown
Galactose	0.89	0.95	1.09	Brown
Lyxose	1.55	1.32	1.28	Red
Arabinose	1.30	1.20	1.34	Red
Xylose	1.37	1.30	1.22	Red
Rhamnose	1.91	1.58	1.60	Brown

Solvents:
a. Ethyl acetate: pyridine: water (8: 2: 1 v/v)
b. 1-Butanol: ethanol: water (40: 11: 19 v/v)
c. Phenol: water (73: 27 w/w)

tirrhinin), cyanidin-3-glucoside (chrysanthemin), pelargonidin-3-rhamnoglucoside and pelargonidin-3-glucoside (callistephin).

Discussion

Acid hydrolysis of anthocyanins purified on Whatman no. 3 filter paper may produce arabinose as an artifact (4). This was eliminated by the use of cellulose powder columns for the resolution of the anthocyanins in the petals of the tulip variety Pride of Haarlem. Separation of the anthocyanins on the cellulose powder columns was usually accomplished within 4 hours and also offered the advantage of being a simple means of purifying a large amount of pigment. The cellulose powder columns had to be remade frequently because of the accumulation of air bubbles. The resolution of the anthocyanins on these columns was not quite as distinct as with filter paper presumably because of the loose packing.

Hall (3) and Beal et al (2) studied the anthocyanins of most species of the genus Tulipa and found that the 2 sub-genera differed in their anthocyanin constitution. Only pelargonidin and cyanidin derivatives were found in the Leiostemones and only cyanidin and delphinidin derivatives in the Eriostemones. The anthocyanin in all cases was a pentoseglycoside. Robinson and Robinson (6) examined 34 varieties of tulips and separated them into 2 groups. They stated that there was 1 group "in which pelargonidin and cyanidin occur as 3-biosides and then apparently with a rather sharp separation a second group containing delphinidin derivatives" sometimes with cyanidins but "they are free from pelargonidins." Apparently a 3rd group can be added: those varieties which contain derivatives of delphinidin, cyanidin, and pelargonidin as found in the variety Pride of Haarlem. Robinson and Robinson (6) also stated that "All [the anthocyanins of the tulip] are diglycosides." On the basis of quantity of pigment in the 2 varieties tested in the present work, the major components were diglycosides, the rhamnoglucosides of cyanidin and pelargonidin. But by the use of chromatographic methods the monoglucosides of cyanidin and pelargonidin were also identified as minor components.

Summary

The anthocyanins in petals of the tulip varieties Smiling Queen and Pride of Haarlem were isolated and identified by chromatographic and spectrophotometric methods. The petal tissue of the variety Smiling Queen contained cyanidin-3-glucoside (chrysanthemin), cyanidin-3-rhamnoglucoside (antirrhinin), pelargonidin-3-glucoside (callistephin) and pelargonidin-3-rhamnoglucoside. The petals of the variety Pride of Haarlem contained the 4 anthocyanins found in the petals of the variety Smiling Queen and delphinidin-3-rhamnoglucoside.

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FORMATION OF EMBRYO STARCH DURING GERMINATION AS AN INDICATOR OF VIABILITY AND VIGOR IN HEAT-DAMAGED BARLEY[1]

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In their classic study of the enzymes involved in the mobilization of stored carbohydrate reserves in barley, Brown and Morris (3), in 1890, noted that the excised barley embryo formed a large quantity of starch when floated on water for a few hours. More recently investigators (2, 4, 5) working with excised barley embryos appear to have ignored or neglected this important biochemical transformation. In this investigation the formation of starch in excised embryos during germination has been studied as a criterion of barley viability and of seedling vigor. Grains of barley were damaged by heating to obtain samples differing in viability and vigor.

MATERIALS AND METHODS

Oderbrucker barley was used in all experiments. Mature grains were placed in a moist atmosphere to facilitate embryo removal. Embryos were removed from the grains with the thumb nail. This technique was found to be more efficient than removal with instruments since less endosperm remained attached to the scutellum. The upper half of the scutellum was usually missing from embryos so excised, thus providing a direct view of the coleoptile. Since the amount of starch formed in the embryo during germination is negligible when compared to the quantity stored in the endosperm, it is of utmost importance to eliminate as completely as possible from the embryo starch determinations the physiologically inert starch of the endosperm.

The embryos, in lots of 100, were floated on 3.0 ml. distilled water in 50 ml. Erlenmeyer flasks, or placed on sheets of Whatman no. 1 filter paper with 4.0 ml. distilled water in Petri dishes at 20° or 25° C.

Germination tests were made at 20° C by following standardized procedures (11). Since the heat-treated grains had to be humidified in order to permit removal of the embryos, all grains were humidified prior to germination tests. After approximately 24 hours in a moist germinator, distilled water was added and the grains were covered with moistened blotters. Germination was determined at 4 to 7 days.

Fresh weight was determined at the end of the 5-day germination period and an average weight was calculated for those seedlings produced from 900 grains. The average fresh weight of the ungerminated grain, roughly equal to that portion remaining attached to the germinated seedling, was substracted from the average fresh weight of the seedlings to obtain a more realistic measure of fresh weight differences. This value represented the increase in seedling fresh weight over that of the ungerminated seed.

Seedling height was measured at 5 days and an average seedling height was calculated. The curve in figure 7 representing seedling height expressed as a percent of the unheated control was obtained from an average of 6 replicates of 100 grains.

Starch formation was determined after boiling the embryos in 80% ethanol and extraction with ethyl ether. Upon the addition of a solution of iodine (2 g/l) and potassium iodide (20 g/l), embryos which stained blue were judged to contain starch.

Quantitative estimates of amylose content were made by grinding the stained embryos in ethyl alcohol in a teflon homogenizer. The samples were then centrifuged. The precipitate was resuspended in water and autoclaved at 15 pounds of steam for 15 minutes. This procedure removed all visible traces of iodine and dissolved the amylose or blue staining fraction of the starch. The sample, colorless after this treatment, was centrifuged to remove debris. Iodine-potassium iodide was added to an aliquot of the supernatant, and the optical density of the solution was measured at 650 mμ in the Spectronic 20 after 15 minutes. After the optical density of the blue iodine-amylose solution was measured, the sample was bleached with sodium thiosulfate and the optical density value obtained (due to suspended inert material) was subtracted from the first reading. This gave a more accurate "blue value" for amylose. The amylose content of a sample was expressed in terms of a Merck water soluble amylose used as a standard; this value is in reality an "apparent" amylose. For reasons of brevity, it is referred to in this paper as amylose content. Because only small quantities of embryo starch were available, the amylose : amylopectin ratio (8) was not determined. This ratio is used to calculate absolute amylose values but absolute amylose values were not considered necessary in this study.

Some starch and carbohydrate determinations were made by the method of McCready et al (9), using perchloric acid and anthrone reagent. Samples were usually analyzed for amylose by the autoclave method described above because of its simplicity and reliability.

Samples of barley grains possessing different capacities for germination were obtained by heating them on 2 shelves in a convection oven at 70°, 80°, 85°, or 100° C for as long as 96 hours. Samples

[1] Received January 12, 1959.

heated at 85° C were used for most of the experiments reported here.

Experimental Results

The time course of occurrence of starch (amylose, which stains blue with iodine) and of total perchlorate soluble carbohydrate (anthrone reagent) in the floated embryos at 20° C is shown in figure 1. Starch formation apparently is fairly rapid after a slight decrease, which may be due to digestion of traces of starch adhering to the embryos, or to physical removal of such starch due to handling. At the end of 18 hours, at which time the experiment was terminated, the amylose content had increased several-fold. The greater increase in perchlorate soluble carbohydrate presumably represented the synthesis of additional carbohydrate polymers such as amylopectin, glucosan, or other hemicelluloses. Amylose values were emphasized in this study because the amylose showed the greatest relative increase during germination and because this substance could be measured by simple procedures. The iodine test for amylose provided a qualitative test for starch in individual embryos which could be compared with germination percentages. After extraction of the amylose from the em-

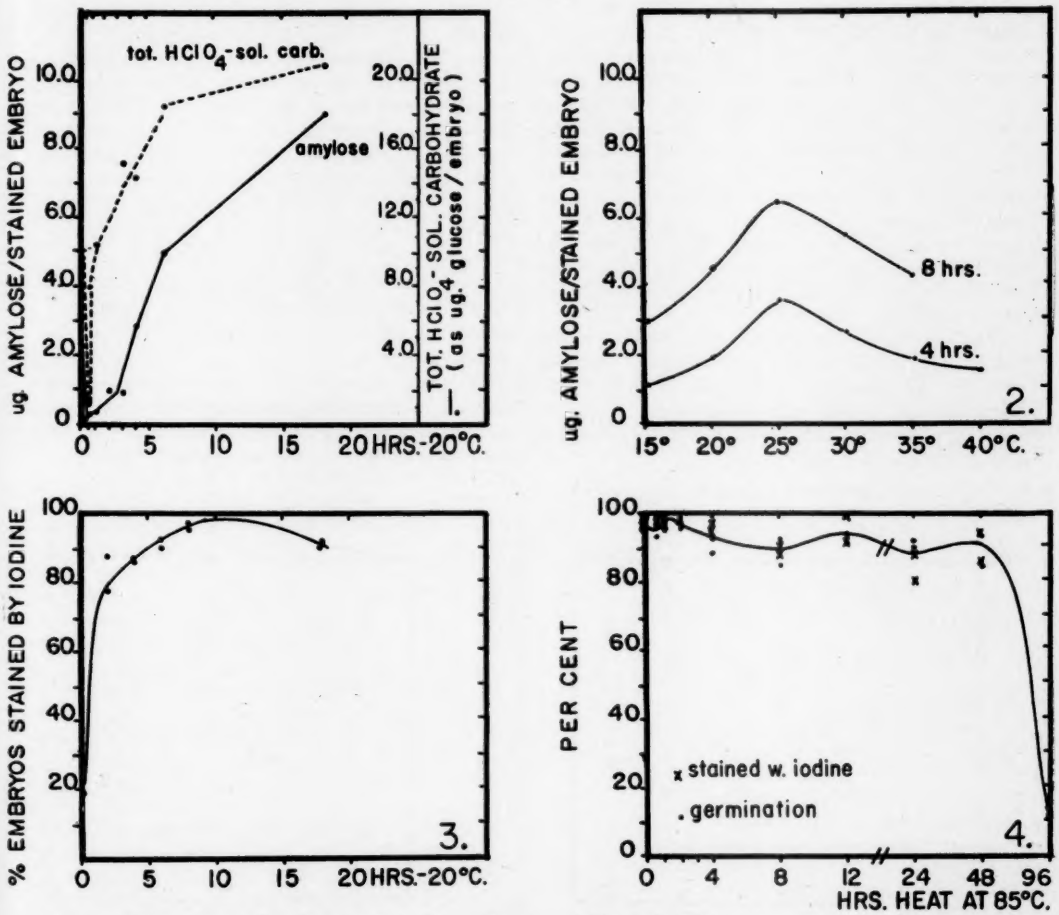


Fig. 1. Time course of amylose and total perchlorate soluble carbohydrate formation in 100 excised barley embryos floated on distilled water at 20° C for time intervals up to 18 hours.

Fig. 2. Temperature curve for amylose formation in 100 excised barley embryos floated on water for 4 or 8 hours.

Fig. 3. The effect of length of time of floating on the percent barley embryos (2 × 100) showing a positive test for amylose.

Fig. 4. Comparison of average percent germination (6 × 100) with average percent embryos (4 × 100) stained by iodine in barley samples heated at 85° C for different time intervals up to 96 hours.

bryos, the iodine test was reapplied (to the extract) for a quantitative measure of amylose content per stained embryo. The latter value was related to vigor. By using the iodine staining procedure, starch formation was recognizable in embryos from vigorous seeds after 2 hours in distilled water.

Figure 3 shows the time course of the percent of embryos staining. Dry embryos stained by iodine were a very dark brown, and some endosperm starch often adhered to the excised embryo. Consequently, the presence or absence of blue stain in such embryos was difficult to detect. Only traces of blue staining material were found upon microscopic examination. After 2 hours growth on water, however, a large percentage of the embryos stained with a greenish coloration, due to the masking of the blue starch-iodine complex by the inherent yellowness of the tissues. At this stage the embryo had enlarged considerably, having absorbed water, and had lost the brown coloration found in the dry unfloated embryo which had been stained with iodine. After 8 hours growth, the number of embryos staining blue reached a maximum of 98 %, approximately that of the actual germination (99 %). At this stage the embryos stained dark green, blue-green, or blue-black, depending on the amount of starch formed. After 18 hours, the embryos had lost most of the yellowness, and had grown a millimeter or two. At this point they stained an intense blue-black with iodine.

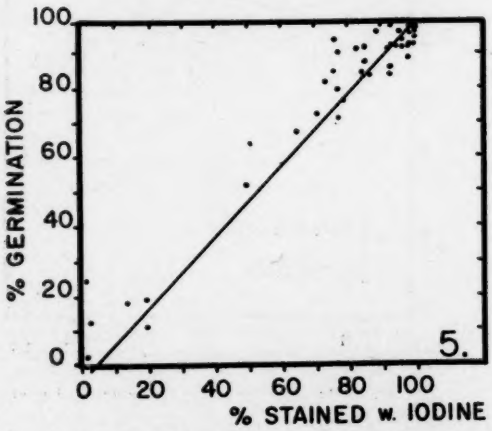
Staining of the coleoptile has been considered the primary criterion for viability in this study. In some immature heat-damaged seeds, the normal viable embryo staining pattern was reversed, the coleoptile remaining yellow or unstained, the scutellar region staining blue.

The optimum temperature for starch formation was determined by floating embryos for 4 or 8 hours at temperatures ranging from 10° to 30° C. The embryos were extracted with ether and stained with iodine. The stained embryos were ground and apparent amylose per stained embryo was determined. Figure 2 shows in 5-degree intervals, the temperature curve for starch formation for 4 and 8 hours. In both instances, maximum starch formation occurred at 25° C.

Many of the earlier experiments with excised embryos were carried out at 20° C, the optimum temperature for germination of the intact seed. In later experiments, excised embryos were grown at the optimum, 25° C, unless otherwise specified.

The germination of a series of barley samples heated at 85° C for different time periods up to 96 hours was determined. The values obtained were compared with the percentages of excised embryos which stained blue with iodine after floating for 8 hours in distilled water. The results are summarized in figure 4. Heating at 85° C for time intervals up to 48 hours had little effect on the percent of germination at 4 days; a marked decrease in germination occurred after 96 hours heating. The percentage of excised embryos that stained blue with iodine was closely correlated with the percent of germination, indicating an intimate relationship in the embryo between the ability to form starch, and the ability to grow.

Figure 5 shows a summary of some 51 determinations in which percent germination was plotted against the corresponding percent embryos giving positive tests for amylose with iodine. The samples



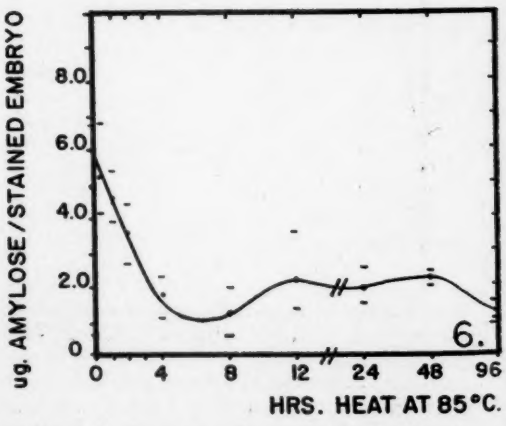


FIG. 5. Comparison of average percent germination (3 × 100) with average percent embryos (1 × 100) stained by iodine in 51 determinations using seeds treated or damaged by heating at 70°, 80°, 85°, and 100° C for varying periods of time; unheated controls are included.

FIG. 6. Average μg amylose per embryo formed in 8 hours at 20° C in iodine-stained embryos from seed heated at 85° C for different time intervals up to 96 hours; average from 4 replicates of 100 embryos per sample.

TABLE I

STATISTICAL TESTS FOR LINEAR CORRELATION OF NUMBER OF EMBRYOS YIELDING POSITIVE TESTS FOR AMYLOSE AND PERCENT GERMINATION; AMYLOSE CONTENT OF EMBRYOS AND FRESH WEIGHT, INCREASE IN FRESH WEIGHT, AND SEEDLING HEIGHT IN HEAT-DAMAGED BARLEY SAMPLES

TEST	CORRELATION COEFFICIENT	DEGREE OF FREEDOM
	r	*df*
Number of embryos stained by iodine vs % germination	0.981**	49
Amylose per stained embryo vs average fresh weight	0.712*	8
Amylose per stained embryo vs increase in fresh weight	0.799**	8
Amylose per stained embryo vs seedling height	0.786**	8

* Significant at the 5 % level.
** Significant at the 1 % level.

consisted of controls and grains heated at 70°, 80°, 85°, and 100° C; length of time of embryo growth was 3, 4, or 8 hours. A highly significant correlation coefficient (10) was indicated between percent germination and percent embryos stained. This is shown in table I.

Observation of the seedlings in these experiments revealed that those from heated grains grew at a slower rate; likewise, the embryos from the heated grains showed a progressively less intense stain for amylose. Figure 6 shows the average apparent amylose formed in embryos of grains damaged by heating. A decrease in amylose content was noted after only 30 minutes of 85° C heat; a further rapid drop occurred up to 4 hours heat, when a plateau was reached. This did not appear to prevent germination, as most of the grains heated up to 48 hours germinated in large numbers. As shown in figure 7, however, the heat treatment did have a depressing effect on height of the seedlings and on fresh weight.

In figure 7 average fresh weight, average increase in fresh weight, and average seedling height at 5 days are compared to average amylose content per stained embryo after 8 hours growth; comparisons are expressed as percent of the unheated controls. It is readily seen that the heat treatment caused a drastic decrease in the ability of the embryo to form starch; this is reflected in a less pronounced, but definite decrease in seedling height and fresh weight. A more striking decrease of the gain in fresh weight over that of the ungerminated grain also is apparent.

Mention should be made, regarding figure 7, of the undulations in the curves in the 8- to 96-hour region. Both the upper and lower shelves of a convection oven had been used for some of the longer heat treatments required in this study. Although it was not realized at the time, the lower shelf supplied a higher temperature (as much as 2° C higher) to the grains. It is of interest to note that the maxima and the minima of the embryo amylose curve are uniformly reflected in the curves for fresh weight, increase in fresh weight, and seedling height. When the amylose values in figure 7 were plotted against the corresponding values of fresh weight, increase in fresh weight, and average seedling height, a significant linear correlation (10) was found to exist in all cases. The correlation coefficients are presented in table I.

DISCUSSION

Several chemical tests have been reported to be useful for making quick estimates of germinability and of viability. Biselenite, triphenyl tetrazolium chloride, and hydrogen peroxide have been used with some success for this purpose; they do not, however, furnish a quantitative quick test for plant vigor or vitality. Furthermore, the tetrazolium derivatives in use thus far appear to be unreliable with certain types of seed samples. MacLeod (7) has investigated this problem using triphenyl tetrazolium chloride, while Bishop (1) has done the same with the iodonitro derivative. In the use of heat for the artificial drying of barley, both MacLeod and Bishop found that a certain series of high grain-moisture contents combined with abnormally high drying temperatures, gave germination values in which the tetrazolium figures were much higher than actual germination tests. Some of the tetrazolium values reported were several times greater than actual germination values. MacLeod (6) in an interesting study of various enzyme activities as related to barley viability, indicated that the dehydrogenase responsible for the reduction

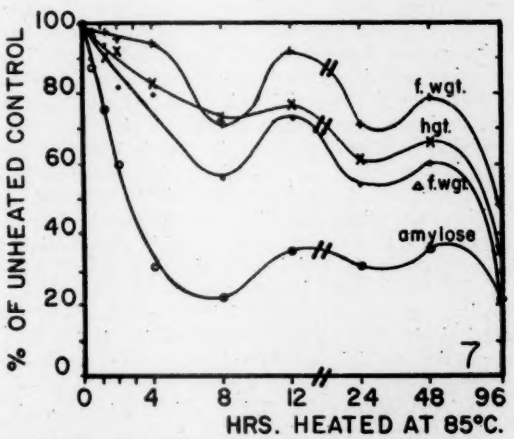


FIG. 7. The effect of heating barley samples for time intervals up to 96 hours at 85° C on the following seedling properties: amylose content per embryo (4 × 100 embryos) after 8 hours growth; seedling fresh weight and increase in seedling fresh weight (9 × 100 seedlings) at 5 days; and seedling height (6 × 100 seedlings) at 5 days. (Results are expressed as percent of the untreated or unheated controls.)

of triphenyl tetrazolium chloride was more stable towards destruction by heat than was the entire germination process. The range of temperatures used in the present experiments to obtain seed lots of different germinative power was in the range where tetrazolium tends to give high estimates of germination. High tetrazolium values were indeed obtained with some of the samples used in the experiments described in this report. Embryo starch determinations, in such instances, gave estimates of germinability which were very close to the actual values.

The sensitivity of the embryo starch test is without doubt related to the mechanism of amylose formation during germination. No information on the specific mechanism of amylose formation from stored reserves in the barley embryo is available. Both carbohydrate and fat have been found in barley embryos in amounts great enough to serve as sources of supply. James (4) showed that the embryo contained large amounts of sucrose and raffinose which were rapidly depleted during germination. James and James (5), although able to show a small decrease in fat content and low R.Q. values, concluded that hemicelluloses, cellulose, and a trace of starch were synthesized from carbohydrate reserves, while fat was oxidized to form cutin and possibly some carbohydrate which accumulated as fibre. Brown (2) using a different technique, reported an R.Q. of 0.8, increasing to 1.0 in a few hours for excised barley embryos. None of the previous investigators measured amylose content or leakage of solutes. Brown (2), however, mentioned that an appreciable amount of the dry weight was lost by rapid leaching of solutes. The author, in attempting to measure the disappearance of fat, found that up to 20 % of the dry weight of the embryos was lost in 8 hours to the water in which they were floated. The residue remaining after evaporation of the water contained in addition to other material (carbohydrate, etc.), an appreciable quantity of oily material which oxidized on standing or heating to a brown ether insoluble pigment. The weight of the residue was only slightly less than the loss in dry weight. No loss in fat in 24 hours could be demonstrated. The problem will be reexamined with due consideration given to the leached solutes. The leaching of solutes undoubtedly is of appreciable importance in determining the disappearance of fat.

The available evidence appears to favor carbohydrate as the probable origin of amylose. If an exclusively carbohydrate transformation is involved here, the sensitivity of the embryo test may be closely related to the activity of glucose phosphorylase and associated enzymes. Definitive evidence is not available to support this view.

Another possible mode of amylose formation is from fat stored in the embryo. Such a mechanism cannot be excluded on the basis of any of the experimental evidence now available. If one may assume that starch does arise directly from the metabolism of fat, then starch is an accumulating end product of this long chain of complicated biochemical transformations. As such, starch may be regarded as an overall measure of the metabolic activity of this system. The presence of starch indicates the active presence of all the enzymes and cofactors necessary for its formation. It thus should indicate that the embryo in which it is found has an active and complete metabolic system and is capable of growth. Starch, as an indicator of viability, would have obvious advantages over the tetrazolium test, which reveals the active presence of only one type of enzyme, the dehydrogenase.

Whatever the mechanism of embryo starch formation may be, the experiments described in this paper indicate, at least for Oderbrucker barley, that starch is an accurate index of viability even in heat-damaged barley. Furthermore, the formation of starch in the embryo may be used not only as an index of viability, but also of vigor. An obvious disadvantage of the starch test in its present state of development is the time and labor required, particularly for the excision of embryos.

It should be mentioned that this study has considered germination in terms of total number of viable seeds, and no attempt has been made to correlate seedling abnormalities with microscopic starch patterns in the embryo. Nor has any attempt been made, as yet, to reevaluate the tetrazolium staining values by microscopic examination of stain patterns in heat-damaged barley.

Summary

It has been shown in Oderbrucker barley subjected to a range of experimentally induced heat damage that amylose formation in the isolated embryo is indicative of viability. The amount of amylose formed in a given time (8 hours) was found to be roughly proportional to the fresh weight of the seedling, to the increase in fresh weight of the seedling, and to seedling height at 5 days.

Short exposures to heat at 85° C caused a decrease in seedling fresh weight and height, and a marked decrease in the formation of amylose in the excised embryo. Long exposures were required to bring about a reduction in percent germination and percent embryos giving positive tests for amylose.

The author gratefully acknowledges the advice of Dr. Roman Kulwich on statistical techniques, and the technical assistance of Germaine La Roche and Leonard Figelman in some of these experiments.

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NITROGEN AND SULFUR CHANGES ASSOCIATED WITH GROWTH UNCOUPLED FROM CELL DIVISION IN CHLORELLA VULGARIS [1, 2]

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The cellular events which lead to growth and division, though highly integrated, can be resolved to the extent that growth may continue independently. Analysis of such uncoupled growth has served to define processes essential for the division of the cell. One biochemical syndrome whose interruption seems to cause uncoupled growth involves the metabolism of sulfur compounds. Nickerson and co-workers (19), for example, have discovered that a metabolic imbalance which results in the maintenance of an inadequate supply of reduced sulfur compounds, such as cysteine, is associated with the transformation of dividing cells of *Candida albicans* into non-dividing, filamentous forms. That sulfhydryl groups are somehow connected with cell division is generally accepted (16, 27), although the precise mechanism is still subject to debate. Methionine, another of the sulfur-containing amino acids, also has been implicated in the division mechanism. Spoerl et al found that this amino acid, as well as several other metabolites, occasioned the development of short, rod-like cells of the fungus *Ustilago sphaerogena*, instead of the long, mycelial-like form (25). Conversely, the growth of *Chlorella vulgaris* can be uncoupled from division by selenomethionine [CH_3-Se-CH_2CH_2CH-(NH_2)COOH], a structural analogue known to competitively antagonize methionine (23). When these cells are cultured in the presence of the analogue and with sulfate as their only source of sulfur, division stops and many of the cells increase in size and dry weight. This uncoupled growth, whose duration depends on the concentration of selenomethionine, is followed by the resumption of exponential divisions as well as a return to normal size and weight. The addition of L-methionine at any time will terminate the uncoupled growth and reinstate exponential divisions. These data indicate that when L-methionine which normally originates from sulfate is antagonized by its selenium analogue, division but not growth is prevented, and that the amino acid may consequently have a biochemical function essential for the division mechanism of the *Chlorella vulgaris* cell.

Little more is known about the nature of the uncoupled growth beyond what has been mentioned above. In this paper changes in nitrogen and sulfur metabolism and O_2 uptake have been examined in order to characterize the giant cells biochemically in greater detail.

MATERIALS AND METHODS

GROWTH CONDITIONS: The strain of *Chlorella vulgaris*, the composition and preparation of media, and most other aspects of growth conditions were identical with those described previously (22, 23). The following changes in procedure were made. A sulfate concentration of 3.1×10^{-3} M was chosen in all experiments, since at this level maximum growth was attained with only a 20% depletion of the total sulfur, and a high specific activity could be effected in experiments with radioactive sulfate. Selenomethionine was supplied at a level of 3.1×10^{-5} M which induced a period of uncoupled growth that lasted about 150 hours under the conditions of these experiments. The initial population density was increased to 0.4 million cells per ml to provide more cells for analysis. Culture tubes were sup-

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ported in a circular glass water bath surrounded by a bank of thirty 14-watt, white fluorescent lamps. The light intensity at the position of the culture tubes was approximately 750 ft-c as measured with a Weston illumination meter, model 756. A mixture of 5 % CO_2 in air was supplied at a rate of about 500 ml/min through a gas mixing device as described by Biale (2). Single dry weight and volume measurements were made because the water bath held a limited number of culture tubes, several of which frequently had to be pooled, particularly during the period of uncoupled growth, to obtain adequate quantities of cells for other analyses.

NITROGEN DETERMINATIONS: A micro-Kjeldahl and Nesslerization assay, based on the method described by Koch and McMeekin (14) was used. Four aliquots of cells that had been washed 3 times with distilled water were analyzed for total nitrogen. TCA-insoluble nitrogen was determined on 4 aliquots of cells suspended in an equal volume of cold 10 % TCA for 0.5 hour at 2 to 5° C. The cells were then centrifuged and washed twice with cold 5 % TCA prior to overnight digestion. The slopes of 8 standard curves, derived from the readings of digested and Nesslerized aliquots of $(NH_4)_2SO_4$ that contained 10 to 60 μg nitrogen, and calculated by the method of least squares, gave an average slope with its standard error of 3.3 $\pm$ .03. This value was used to calculate the nitrogen contents of the unknown samples from their Klett readings. The nitrogen per cell values are plotted with their 95 % confidence intervals (standard error $\times$ t value).

OXYGEN UPTAKE: Standard Warburg technique was used to measure the endogenous respiration at 25° C of the uncoupled and normal Chlorella cells.

SULFUR DETERMINATIONS: Radioactive sulfur measurements and fractionations were performed as described by Roberts et al (20), and by Atkinson and McFadden (1). The filter apparatus used for the determination of cellular sulfur was generously supplied by Dr. Dean B. Cowie of the Carnegie Institution of Washington.

After filtration the cells were washed 3 times with sulfur-free medium for total nitrogen determinations or with 5 % TCA for the protein determinations. For the TCA-insoluble sulfur, aliquots of cells were first suspended in an equal volume of 10 % TCA at 2 to 5° C for 0.5 hour.

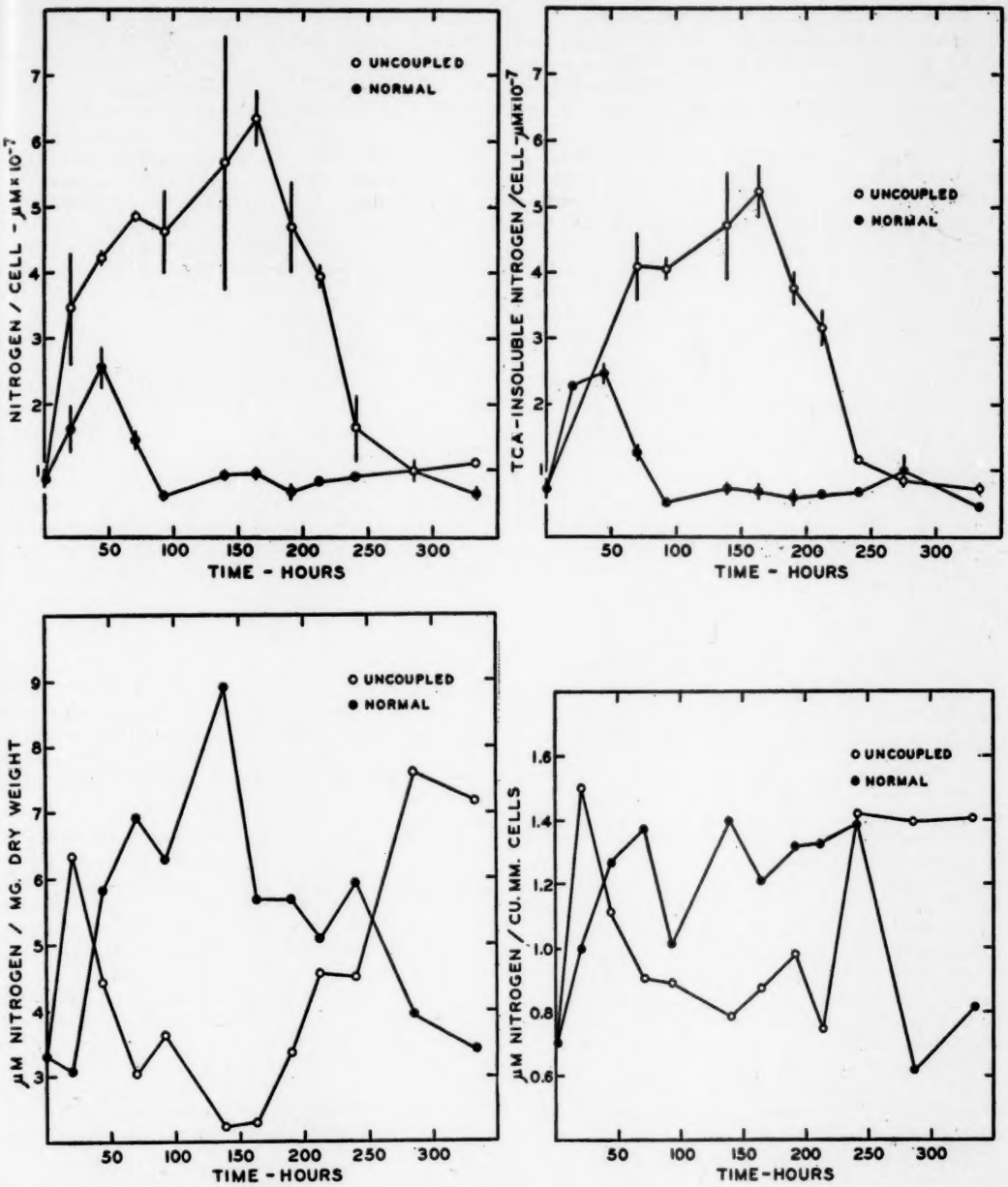
The following fractionation procedure, based on that described by Roberts et al for *Escherichia coli* ((20), p. 13), was adopted. In each of the steps 10 ml of solvent were used. Where evaporation had occurred, the volume was adjusted after decantation. Known numbers of cells, washed 3 times with sulfur-free medium, were suspended in 10 ml of 5 % TCA at 2 to 5° C for 0.5 hour, centrifuged, and the supernatant decanted. The cells were next suspended in 10 ml 75 % alcohol for 30 minutes at 40 to 50° C. A 15-minute alcohol-ether extraction at the same temperature was followed by extraction with 5 % TCA in a boiling water bath for 0.5 hour. A 1 % HCl-alcohol wash for 5 minutes at room temperature followed, and the residue was then thoroughly suspended in 75 % alcohol. Aliquots were withdrawn for counts of the remaining activity in the cells. Centrifugation yielded the final precipitate which is termed the residual protein sulfur and which was eventually analyzed for amino acid content. The supernatant fractions collected in each of the above steps were prepared for radioactivity measurement as described by Roberts et al ((20), p. 9). Throughout the procedure samples for counting were prepared in duplicate.

Counts were made with a Nuclear model 163 scaler equipped with a counter whose mica end-window was approximately 1.4 mg per cm^2. Calculations were corrected for geometry, self-absorption and background. Since the ratio of radioactivity to the molarity of the sulfate in the medium was known, the specific activity was used to express all calculations on a molar basis.

CHROMATOGRAPHY: Residual protein fractions were hydrolyzed in 6 *N* HCl for 4 hours in sealed glass tubes at 105°C ((20), p. 25). The hydrolysates were evaporated to dryness under vacuum at 30 to 40° C, resuspended in water and chromatogrammed. For the two-dimensional chromatograms the solvents consisted of *n*-butanol : glacial acetic : water, 100 ml : 22 ml : 50 ml ((26), p. 36), and methanol : *n*-butanol : benzene : water, 100 : 50 : 50 : 50 (15, 21). After development the Whatman no. 1 sheets were placed on Kodak No-screen x-ray film for varying time periods. The films then were processed and compared with the ninhydrin-sprayed chromatograms.

RESULTS AND DISCUSSION

GROWTH RELATIONSHIPS: Growth patterns were qualitatively similar to those observed in earlier experiments (23) but differed quantitatively in several respects. A maximum dry weight of 29 $\times$ 10^{-8} mg/cell and a maximum volume of 74 $\times$ 10^{-8} mm^3/cell were recorded toward the end of the 150-hour period of uncoupled growth. These data are in contrast to those of the earlier experiments in which cells were exposed to half the concentration of selenomethionine. Previously, uncoupled growth lasted only about 100 hours, and the maximum weights and volumes attained were 17 $\times$ 10^{-8} mg/cell and 60 $\times$ 10^{-8} mm^3/cell. Since these measurements are made on centrifuged, packed cells which consist of a mixture of giant as well as small cells, one cannot tell whether the higher maxima resulted from the development of a larger proportion of giant cells at the higher level of selenomethionine or from a continued enlargement of the same number of giant cells. In the present work a differential count showed that at the end of the period of uncoupled growth approximately 50 % of the cells were larger than 12 μ in diameter, whereas in normal cultures, the maximum diameter of cells during early exponential growth rarely exceeds 10 μ.



Nitrogen changes in uncoupled cells of *Chlorella vulgaris*.

FIG. 1. (*upper left*). Total nitrogen content expressed on a per cell basis.

FIG. 2 (*upper right*). Protein nitrogen content expressed on a per cell basis.

FIG. 3. (*lower left*). Data of figure 1 expressed on a dry weight basis.

FIG. 4 (*lower right*). Data of figure 1 expressed on a packed cell volume basis.

NITROGEN METABOLISM: The changes in nitrogen content of uncoupled cells and of untreated cells are shown in figure 1. The nitrogen contents of the control cells represent average values for a heterogeneous population of cells in all stages of growth and division. Studies of randomly dividing cells give little information about a single cell during its cycle of growth and division. One must resort either to techniques that result in synchronization, so that the majority of the cells grow or divide at the same time (6), or to the study of organs and tissues whose cells show such a synchrony under normal conditions of development (9, 27). In synchronized or uncoupled cultures of microorganisms, moreover, the cells soon reach different physiological ages than the controls, so that a comparison of the 2 types of cultures at any given time after inoculation has only limited meaning. In the Chlorella cultures, for example, at 100 hours the control had reached the end of its exponential phase of growth, and due to mutual shading of the cells, changes in pH, changes in salt concentration, etc., the cells were physiologically different from the giant cells which were still in their uncoupled phase of growth and still enlarging. For these reasons, no detailed comparison has been made between uncoupled and control cells. The important changes in the uncoupled cells are the ones that begin at the time of initial exposure to selenomethionine.

Under the influence of selenomethionine the uncoupled cells continued to absorb nitrates and to incorporate a large portion into protein (figs 1, 2). That the continued uptake and incorporation did not keep pace with the change in size and dry weight is illustrated in figures 3 and 4 in which the same data are plotted on a concentration basis per unit of dry weight and volume. During the 1st 20 hours of exposure to the seleno-analogue there was a sharp rise in cellular nitrogen which may have been due to the rapid uptake of the amino acid in addition to the uptake of nitrate. A steady decline in nitrogen concentration per unit weight and volume occurred for the remainder of the uncoupled growth period. Since the nitrogen content per cell had risen, there must have developed an imbalance between the rates of protein synthesis and the rates of other syntheses which lead to dry weight increase.

Chlorophyll synthesis appears to have been impaired too, judging from the pale green of the packed uncoupled cells. Whether the large increment in dry weight was due to the functioning of pre-existing chlorophyll or to a limited production of the pigment is not known.

At the end of approximately 150 hours, when the giant cells were resuming division, and cell weight and size were falling precipitously to the values of control cells, the nitrogen level per cell also fell rapidly (fig 1). This fall undoubtedly represents a partitioning of the existing nitrogen among the daughter cells and not a slackening in protein formation, since the dry weight and volume relationships (figs 3, 4) show that the nitrogen per unit weight and volume rose to the level of control cells that had reached the end of their exponential growth. Because the newly dividing cells were not synchronized, the data here again represent values for a heterogeneous population of cells. The rise in nitrogen concentration probably was not due to a decline in other syntheses that lead to dry weight increase, for concomitant with new division the cells became dark green again. Instead, the observed rise seems to have resulted from a resumption of the normal rates of protein formation.

Synthesis of cell material in *Chlorella vulgaris* during cell enlargement in the absence of division has also been observed by Finkle and Appleman (10, 11). Magnesium exhaustion from the medium was responsible for the halt in multiplication, in this instance, and increases in dry weight and nitrogen content of the enlarging cells were taken as indices of continuing metabolism.

The cells of the control population in the present studies exhibited an initial rise in nitrogen content (figs 1, 2) which attained its maximum very early during exponential growth. After a rapid drop, a nitrogen level was reached that remained constant. A similar pattern was detected by Fowden in *C. vulgaris* cells grown with nitrate and ammonium salts (12). When the data are expressed on a concentration basis (figs 3, 4) they show that the nitrogen per unit volume or per unit weight of packed cells continued to rise until exponential growth had ceased, and that the nitrogen level was maintained well into the stationary phase of growth. These quantitative changes associated with randomly dividing cultures show the necessity for studying cells along their entire growth curve rather than at some arbitrarily chosen time.

OXYGEN UPTAKE: As another index of metabolic activity, the O_2 uptake of uncoupled cells was measured. Table I summarizes the data obtained with 44-hour-old cells, and although these data do not give a complete picture of the changes throughout the course of uncoupled growth, it is evident that the respiration of the giant cell rose markedly. By 44 hours cell size had increased 6-fold, cell dry weight 4-fold, protein nitrogen 4-fold, and O_2 consumption per cell had tripled. Once again, these data show that the various processes had not kept pace with each other. Unanswered by the data is whether the higher O_2 uptake resulted from continued synthesis of respiratory enzymes, or from a more rapid operation of the existing

TABLE I

OXYGEN UPTAKE BY NORMAL AND BY UNCOUPLED CELLS OF CHLORELLA VULGARIS

AGE OF CELLS HRS	$\mu\mu$L/HR × CELL	μL/HR × MG DRY WT	μL/HR × MM^3 PACKED VOL	μL/HR × μG PROTEIN N
0 (inoculum)	0.176	5.47	2.18	0.160
44 normal	0.345	8.19	1.46	0.105
uncoupled	0.518	4.14	1.08	0.114

enzymes. The increase in protein nitrogen as the uncoupled cell enlarged would presuppose the maintenance of enzyme synthesis, unless these proteins were only structural. If the newly synthesized proteins are indeed enzymes then one is faced with the fact that they lack methionine (see next section). Altered proteins, however, are known, for Gross and Tarver (13) have isolated ethionine from the proteins of Tetrahymena cells that had been grown with this analogue; Munier and Cohen (18) found that β-F-phenylalanine partially replaced phenylalanine and tyrosine in the proteins of *Escherichia coli;* Yoshida (31) has reported the isolation, from a methionine-requiring mutant of *Bacillus subtilis,* of an α-amylase that not only contained ethionine but which also had the same activity as the unaltered enzyme; and there is strong evidence that selenomethionine can be built into the proteins of *Escherichia coli* in place of methionine (7, 8, 28).

SULFUR METABOLISM: The changes in sulfur content and concentration were similar to those of nitrogen. The uncoupled cells absorbed considerable sulfate (fig 5), although not in proportion to the increase in dry weight (fig 6) or size. This sulfate was metabolized into a variety of fractions, as shown in table 2 which lists the distribution at 92 hours of the sulfur in giant cells as well as in randomly dividing cells. The giant cells incorporated sulfur into the same fractions as did the normal cells, with a large amount soluble in hot TCA which is commonly used to extract nucleic acids. In comparable studies with *E. coli* ((20), p. 320), in which the technique for radioactive fractionation was essentially the same as the one used here, very little radioactive sulfur was removed by hot TCA. The nature of this sulfur component in *Chlorella vulgaris* has not yet been characterized. A portion of the total sulfur found its way into the residual proteins, but radioactive methionine could not be detected in the acid hydrolysate (figs 7, 8). However, the sulfur amino acid was again found in the protein hydrolysate of cells that had resumed division (fig 9). The renewed ability to incorporate methionine into protein may have some bearing on the ability of these cells, when subcultured into fresh medium that contains selenomethionine, to divide after only a short period of uncoupled growth.

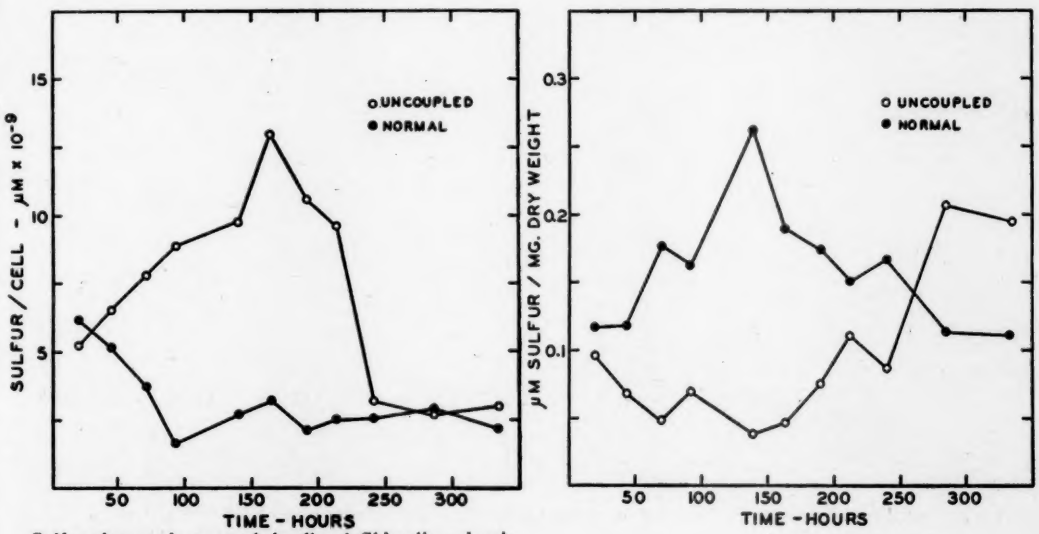
TABLE II

SULFUR FRACTIONS IN NORMAL AND IN UNCOUPLED CELLS OF CHLORELLA VULGARIS *

	SULFUR CONTENT PER CELL **			
	NORMAL CELLS		UNCOUPLED CELLS	
	$\mu M \times 10^{-12}$	% of total	$\mu M \times 10^{-12}$	% of total
Total sulfur	1659	100	8914	100
Cold TCA soluble	308	18.5	1747	19.6
75% ethanol soluble	138	8.2	739	8.3
Alcohol-ether soluble	13	0.8	65	0.7
Hot TCA soluble	298	17.9	2313	26.0
Acid alcohol soluble	28	1.7	110	1.2
Insoluble (residual protein)	856	51.5	3432	38.5
	Recovery	98.6%		94.3%

* Age of cultures, 92 hrs.

** Specific activity of culture medium: 6147 cts/min × μM sulfate for normal; 6818 cts/min × μM sulfate for uncoupled; cold sulfate conc, 3.1×10^{-3} μM/ml.

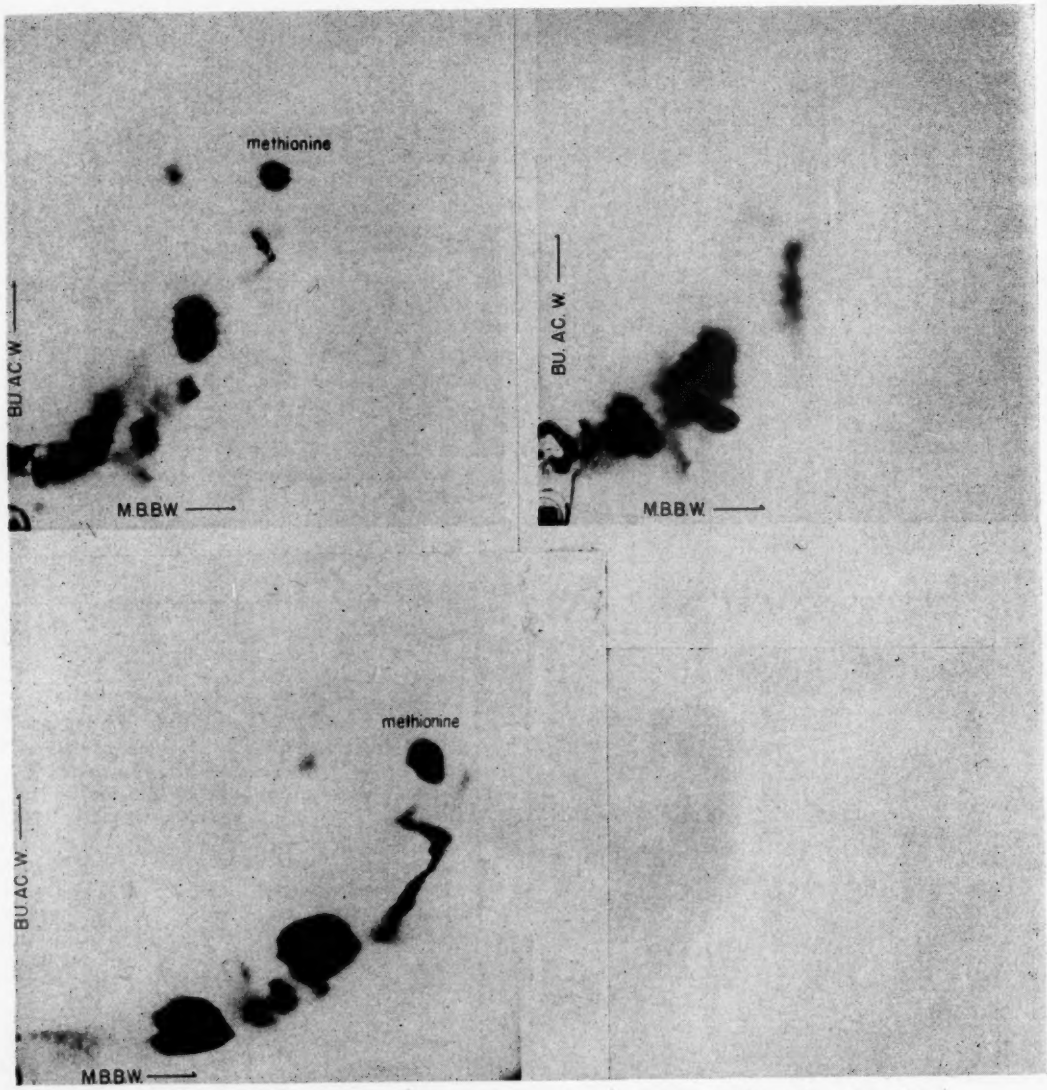


Sulfur changes in uncoupled cells of *Chlorella vulgaris.*

FIG. 5 (*left*). Total sulfur content per cell.

FIG. 6 (*right*). Data of figure 5 expressed on a dry weight basis.

The absence of radioactive methionine in the proteins of the uncoupled cell is consistent with other work in which selenomethionine was shown to compete with methionine. A competitive relationship between the 2 molecular species was first established in growth and absorption studies with *C. vulgaris* (23). In these experiments the exponential rate of growth and the selenium content of the cells were a function of the external methionine/selenomethionine ratio, an indication that the analogue pair had competed for passage across the cell membrane as well as for an enzyme site within the cell. More recently Cowie and Cohen (7, 8) confirmed such a competition by the demonstration that selenomethionine decreased the incorporation of radioactive methionine into the proteins of a methionine-requiring mutant of *Escherichia coli*. Furthermore, the selenium analogue supported exponential growth of the mutant in the



Radioautographs of protein hydrolysates of ***Chlorella vulgaris*** cells grown with radioactive sulfate.
FIG. 7 (*upper left*). Normal Chlorella cells.
FIG. 8 (*upper right*). Uncoupled Chlorella cells. Note absence of methionine.
FIG. 9. (*lower left*). Chlorella cells that have resumed exponential divisions after uncoupled growth.

complete absence of the sulfur compound. The conclusion from these data, that selenomethionine can be built into proteins in place of methionine, is borne out by the experiments of Tuve and Williams (28) with wild type *E. coli,* in which radioactive selenomethionine was identified in the proteins after the cells had been exposed to radioactive selenite.

Additional evidence for a competition can be derived from the relationships between other selenium compounds and the analgous sulfur-containing metabolites. Selenocystine prevents the influx of S^{35} L-cystine into leukemic leukocytes and into Murphy lymphosarcoma cells of the rat (29, 30), and a competitive antagonism between the sulfate and selenate ions has been demonstrated in many biological systems (22, 24). There appears to be a general competitive relationship between many sulfur metabolites and their selenium analogues.

One can be reasonably certain that in the mutant studied by Cowie and Cohen, and in the uncoupled Chlorella cell, selenomethionine has been built into proteins and probably into functioning enzymes. But the critical experiment to show that an isolated enzyme, altered in this way, can still function has not yet been performed.

Incorporation into proteins is, however, not the only biochemical reaction in which selenomethionine and methionine can compete. Mudd and Cantoni have demonstrated that the enzyme system which transforms methionine into S-adenosylmethionine will also operate as well, if not more rapidly, with selenomethionine as substrate (17).

This last discovery raises the question of the biochemical site at which selenomethionine acts to prevent cell division. Methionine apparently has at least 2 general functions in organisms, one as a constituent of proteins, although some proteins have been isolated which seem to lack methionine ((3) pp. 252–337), the other as a methylating agent. In higher plant cells, more specifically, methionine has been implicated as a source of methyl groups for lignin (5) and for certain alkaloids (4). In Chlorella, however, a methylating role for methionine has not yet been established.

In an overall evaluation of the uncoupling effect induced by selenomethionine a possible transformation of this analogue into other selenium compounds must be considered. Since *C. vulgaris* cells can satisfy all their sulfur requirements with either D- or L-methionine, the enzymes that are involved in the utilization of these substances most probably also metabolize the DL-selenomethionine into other forms which conceivably might be more directly connected with the uncoupling. In addition, it must be recognized that secondary reactions that result from the metabolic derangement caused by selenomethionine or its products may be responsible for the uncoupling. As a first approximation though, the uncoupled growth appears to be correlated with the synthesis of altered proteins which may be incompatible with division.

Summary

The course of metabolic changes was analyzed in cells of *Chlorella vulgaris* that were exposed to selenomethionine whose uncoupling action allowed cell growth to continue without division. The giant cells continued to synthesize protein and to incorporate radioactive sulfur, derived from sulfate, into the protein as well as into a number of other fractions. Radioactive methionine could not be detected in the protein fraction. The increase in protein did not keep pace with the increase in cell weight and volume. The pale green of the packed giant cells indicated impairment to chlorophyll synthesis, in spite of which the dry weight of the uncoupled cells rose markedly. Oxygen uptake by the uncoupled cell tripled after 44 hours of growth in the presence of the seleno-analogue, but the rise was not in proportion to the increase in weight, volume, or protein.

With the return of exponential divisions after the period of uncoupled growth, the cells assumed the characteristics of normal Chlorella cells. Size, weight, protein nitrogen and protein sulfur reverted to the usual levels, and methionine could once more be detected in the proteins.

The author wishes to thank Miss Eva Konrad for technical assistance, and Mr. Michael Luskin for his help with the nitrogen assays.

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TRANSLOCATION OF CALCIUM IN THE BEAN PLANT [1, 2, 3]

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There are a number of reports in the literature of translocation which indicate that calcium is immobile in the phloem, or relatively so (1, 2, 4, 5, 13). The majority of these are not quantitative studies but merely indicate that calcium translocation compares poorly with that of phosphorus, sulfur, potassium and some other elements. Contrasted with these reports on calcium immobility in the phloem are two which claim that the anesthetizing of leaves with diethyl ether (6) and the application of triiodobenzoic acid (TIBA) (8) cause a reversal of polarity in the phloem and thereby allow calcium to move from leaves. However, neither of these papers established that the calcium transport from the leaf was via the phloem.

The purpose of the present investigation is therefore 2-fold: to examine calcium translocation in more quantitative terms, and to investigate the effects of certain substances or treatments on calcium translocation. In addition we have carefully determined the tissue through which calcium movement occurs.

The quantitative study was made by appraising the mobility of cotyledonary calcium, by observing the partition of the calcium absorbed from the nutrient medium between various plant parts, by observing the

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[3] The radioisotopes were acquired from the U.S.A.E.C., Oak Ridge, Tennessee.

subsequent exchange of the calcium between these parts, and finally, by making quantitative measurements of the export of foliarly applied calcium to other parts of the plant.

To accomplish the second part of the study we have investigated the effect on calcium transport of ether and TIBA, substances claimed to reverse polarity, and also the effect of NaCl since in our laboratory we have observed that foliar sprays of high salt content induce movement of calcium from leaves under certain conditions.

Methods

General: Red Kidney bean plants were grown in a Hoagland-type nutrient solution which was changed each 4 days. A ½ strength solution was used during the first 4 days and subsequently a solution of 2.5 mM $Ca(NO_3)_2$, 2.5 mM KNO_3, 1.0 $MgSO_4$, 0.25 mM KH_2PO_4, and with Fe and micronutrients. The solutions were aerated and held near pH6. The growth conditions were: temperature 23 ± 1° C, relative humidity 60 ± 5 %, and light at 1000 to 1200 ft-c (fluorescent) on a 12-hour photoperiod. The special conditions of each of a rather large number of experiments are listed separately.

Partition of Available Calcium: As the young plants developed they had a potential source of calcium from the cotyledons and they were given various additional amounts of calcium via the nutrient medium. Plants survived when grown between the nutrient levels of 0.05 and 5.0 mM Ca. Beyond these limits a deficiency or a chlorosis developed which resulted in the death of the plants. $CaCl_2$ or $NaNO_3$ was used to obtain calcium levels different from 2.5 mM without varying the concentration of essential nutrients Plants were grown to an age of 12 days from the straightening of the hypocotyl. Six plants in 6 liters of solution served as a unit, and the composited similar parts of each 6 plants constituted the samples for analysis. Experiments were in duplicate and determinations were by flame spectrophotometry. The data are compiled in figures 1 A and 1 B.

Exchange of Calcium Between Plant Parts: The exchange of calcium was shown by use of the Ca^{45} isotope. All plants received 2.2 μc of Ca^{45} per liter of nutrient solution between the 4th and 8th days from the straightening of the hypocotyl. Immediately after the 8th day 6 plants were harvested for radioactive analysis and 3 for radioautograms to determine the initial distribution of the Ca^{45}. A similar group of plants was transferred to a non-radioactive complete nutrient solution and held for 4 additional days after which 6 plants were harvested for radioactive analysis and 3 for radioautograms. The final distribution of Ca^{45} compared with the initial distribution indicated the amount of exchange between parts. Plants used for radioautograms were quickly dried between blotters in a forced draft oven at 85° C, then exposed to no-screen x-ray film. Repeated tests have shown that no artifacts in calcium distribution occur as the result of the drying procedure unless the leaf is injured by high salt content sprays, TIBA sprays, anesthetization with ether, and presumably other means (see below).

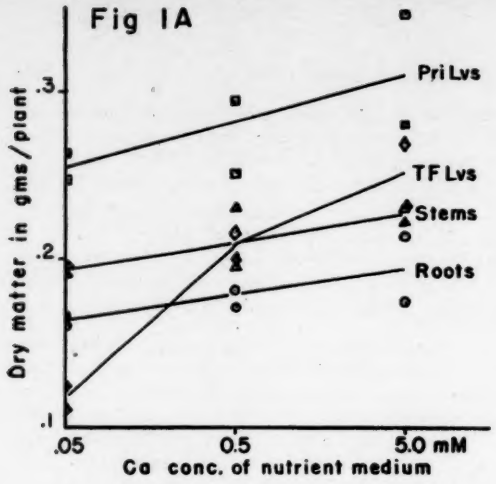
Ca^{45} was determined quantitatively in the calcium oxalate precipitate from the ashed samples; then the total calcium was determined by volumetric analysis. All radioactive analyses were corrected for background, geometry, decay, and self absorption when applicable. Data are represented in figures 2 and 3.

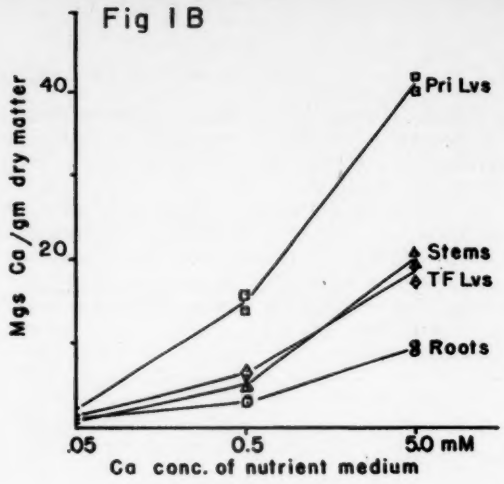
Foliar Export of Calcium: All studies were based on the foliar application of Ca^{45} and its subsequent detection in other parts of the plant by radioactive analysis or radioautography. The tracer applications were all made to the lower surface of the terminal leaflet of the 1st trifoliate leaf. A glass sprayer delivered the solution to an area approximately 1.5 inches in diameter which was centered laterally and located between one fourth to one third of the distance from the base to the tip of the leaflet. The pH of the solutions applied was near 4.0. The migration periods were 24 hours in all cases except those performed to establish the time sequence for the appearance of tracer calcium in the stem.

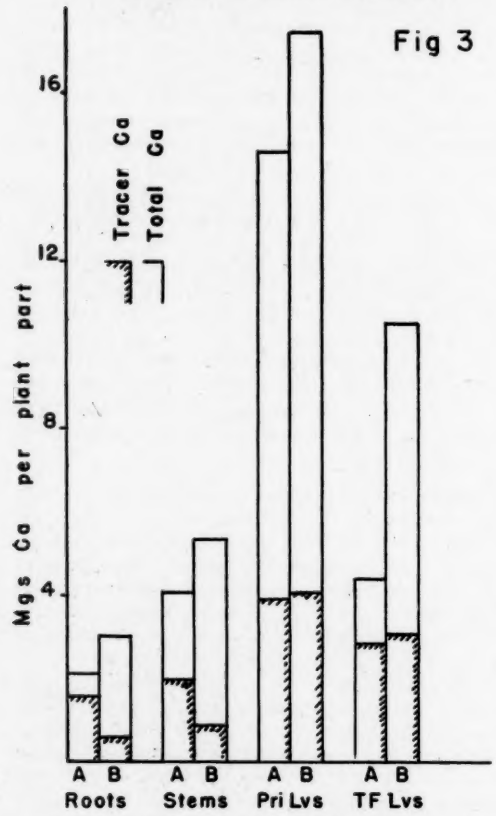
A quantitative evaluation of calcium translocation was made for each of several levels of foliar applied tracer calcium. The foliar spray consisted of 20 μc of Ca^{45} in 50 μl of $CaCl_2$ solution varying in concentration from 0.01 to 1.0 M. Following the 24-hour migration period each plant part was analyzed separately for Ca^{45}. The total Ca^{45} and the percentage of the applied Ca^{45} which was translocated was then calculated. The conventional procedure of steaming the petiole to restrict phloem movement was used to establish the tissue through which movement occurred. The data are compiled in figure 4.

Attempts to Increase Foliar Export: *1. Nutrient treatments*: It was our intent to determine whether or not the foliar export of calcium could be influenced by nutritional conditions. The effects of high and low internal concentrations of certain nutrient elements, and of pH, were investigated. Groups of plants grown under all 8 possible combinations of nutrient conditions resulting from calcium at 0.5 and 5.0 mM, phosphorus at 0.05 and 5.0 mM, and hydrogen ion concentrations at pH 4.0 and 7.0 served as experimental material. A second experiment similar to the above was conducted in which sulfur at 0.01 and 1.0 mM was a variable instead of phosphorus. This made a total of 16 different treatments. Twelve days after the straightening of the hypocotyl a foliar application of approximately 50 μl of 0.2 M $CaCl_2$ containing 16 μc of Ca^{45} was made. Following the 24-hour migration period the plants were dried and radioautograms prepared. The experiment was conducted in duplicate with 6 additional replications at low Ca, low P, and pH 4.0. The results showed no significant differences between treatments so the radioautograms, 38 in all, are not included here.

2. Foliar Treatments: An investigation was made of the effects of NaCl, TIBA and ether treat-







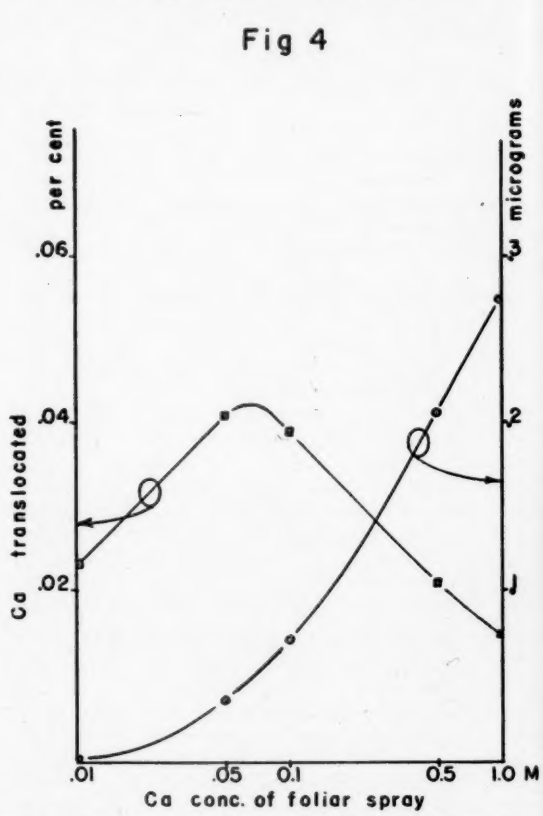


FIG. 1 A. The dry matter yield of different plant parts in relation to the calcium concentration of the nutrient medium. FIG. 1 B. The calcium concentration of different plant parts in relation to the calcium concentration of the nutrient medium. The plant was divided as follows: Primary leaf blades (Pri Lvs), trifoliate leaf blades (T F Lvs), stem including hypocotyl and petioles (Stems), and roots (Roots).

FIG. 3. Histogram showing the distribution of tracer calcium (and total calcium) at 2 intervals following application to the root via the nutrient solution. A. Distribution immediately following an application between the 4th and 8th day from the straightening of the hypocotyl. The nutrient solution contained 2.2 μc of Ca^{45} per liter per plant. B. Distribution at the end of the 12th day. From the 8th to the 12th day the plant was in a normal (nonradioactive) nutrient solution.

FIG. 4. The amount of tracer calcium translocated from a leaf in 24 hours shown in relation to the concentration of the foliar spray applied to the leaf. The foliar spray consisted of 20 μc of Ca^{45} in 50 μl of $CaCl_2$ solution varying in concentration from 0.01 to 1.0 M. The percent of the applied tracer translocated is shown on the left; the total amount translocated on the right.

ments on calcium export from a leaf. The details of each treatment follow:

a. NaCl: A foliar spray of 33 μc of Ca^{45} in 50 μl of 0.03 M $CaCl_2$ and 1.18 M NaCl was applied to the lower surface of the terminal leaflet of the 1st trifoliate leaf. Following the 24-hour migration period plants were either dismembered before radioactive analysis or dried intact and then analyzed. The analyses did not include the treated leaf. The data are compiled in figure 5.

b. TIBA: Seven ml of a TIBA spray at 100 ppm containing 0.05 % Tergitol-7 surfactant was applied to completely wet the aerial parts of each plant. Thirty minutes later a foliar spray consisting of 33 μc of Ca^{45} in 50 μl of 0.015 M $CaCl_2$ was applied. The experiment was otherwise similar to part a. above.

c. Diethyl ether: One minute after a foliar application of 33 μc of Ca^{45} in 50 μl of 0.015 M $CaCl_2$ was made, the treated leaflet was enclosed in a transparent leaf chamber containing liquid ether in a Petri dish. The experiment was otherwise similar to part a. above.

As the ether treatment was the only one which induced an abnormal calcium movement to occur during the migration period, a refined ether treatment procedure was developed in order to determine the tissue through which movement occurred: One minute after a foliar application of 32 μc of Ca^{45} in 50 μl of 0.384 M $CaCl_2$ the treated leaflet was inclosed in a transparent leaf chamber of 2 liters capacity. Air, containing ether vapor, was circulated through the chamber at the rate of 250 ml per minute for 8 minutes. The 2000 ml of air contained the vapor from 1.88 ml of liquid ether. Following an 8-minute ether treatment period a subsequent migration period of either 6, 11, or 22 minutes was allowed during which time the ether-laden air was replaced by moisture-laden air circulated at the rate of 1 liter per minute. Immediately following the migration period, the stem for a distance of 3 inches above and 5 inches below the node of the treated leaf was cut into 1-inch sections, the bark stripped from the wood, and each part analyzed separately for Ca^{45}. The experimental conditions resulted in only a very slight flagging of

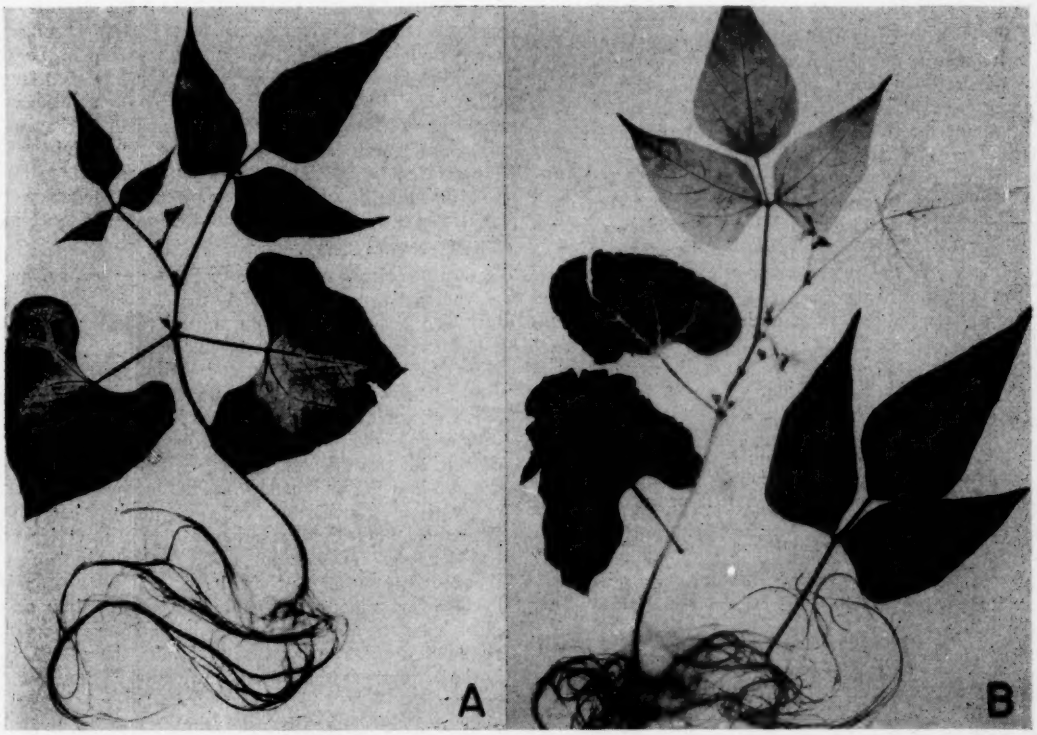


FIG. 2. Ca^{45} radioautograms showing the distribution of the labeled calcium at 2 intervals following application to the root via the nutrient solution. A. Distribution immediately following an application between the 4th and 8th day from the straightening of the hypocotyl. The nutrient solution contained 2.2 μc of Ca^{45} per liter per plant. B. Distribution at the end of the 12th day. From the 8th to the 12th day the plant was in a normal (nonradioactive) nutrient solution. One primary leaf and the 1st trifoliate leaf have been rearranged to fit on the film. The primary leaves of B were injured during the preparation of the radioautograms. Exposure to no-screen x-ray film 60 days.

the ether treated leaf. If the treatment exceeded 8 minutes the leaf collapsed and the results showed that this was invariably followd by a rapid and marked movement of Ca^{45} from the leaf. The data are recorded in table I. Photomicrographs of stripped bark and wood are shown in figure 6. "Bark," as herein used, refers to all tissues exterior to the cambium (7) and "wood" to all tissues interior to the cambium. Under the above growth conditions a well-developed cambium is formed so that stripping is easily accomplished.

Results

Partition of Available Calcium: The relationship between the dry matter yield and the logarithm of the calcium concentration of the nutrient medium was approximately linear for all plant parts. The increment gain in dry matter per unit increase in the logarithm of the nutrient calcium concentration was slight for primary leaves, stems and roots. The high intercept of these curves on the axis representing the lowest calcium concentration which could support growth of the trifoliate leaf system indicated that the early establishment of root, hypocotyl, and primary leaves occurred principally on cotyledonary calcium. For the growth of the trifoliate leaf system an external source of calcium was clearly necessary. This is distinctly indicated by the very meager growth of these parts at the 0.05 M calcium level and the very pronounced gains as the amount of available calcium was increased (fig 1 A).

The primary leaves accumulated and retained a higher concentration of calcium than any other plant part. This was true at each level of calcium nutrition (fig 1 B). The concentration in the stems and trifoliate leaves was intermediate while that of the roots was lowest. These results suggest that the leaves with the longest history of accumulation have the highest calcium concentration since it is not withdrawn in significant amounts for use elsewhere.

Exchange of Calcium Between Plant Parts: Once labeled calcium is deposited in leaves, following a particular absorption period, a significant quantity is not withdrawn to supply leaves formed at a subsequent period. It then follows that the growth of the plant is immediately dependent on the current acquisition of calcium from the growth medium; little, if any, is mobilized from previous depositions. This is evident from a comparison of the Ca^{45} radioautograms in figure 2 A and 2 B.

The same conclusions are supported by the analytical data of figure 3. Here it is shown that the trifoliate leaves which developed during the last 4 days when no tracer calcium was available in the nutrient solution gained only 0.3 mg of labeled calcium from the remainder of the plant while making a gain of 6.2 mg of calcium from the nutrient medium. It is probable that a part of the 0.3 mg gain, if it is significant at all, was from labeled calcium in transit within the plant when the shift to unlabeled calcium was made.

A loss of labeled calcium from root and stem during the later growth period is evident from figure 3. In the root this loss can be explained best by assuming an exchange of the labeled calcium within or upon the root for the unlabeled calcium in the nutrient medium. The loss from the stem, if it is significant at all, must also be explained by this same mechanism since the labeled calcium did not move into the leaves. However, it is only the movement of calcium to the root, bringing it into a position for exchange, and not the exchange itself which would be of significance in the present investigation.

Foliar Export of Calcium: When the amount of radioactive tracer calcium translocated from the leaf was plotted against the molarity of the applied foliar spray, it became evident that the two were related and that calcium translocation was quantitatively measurable. These results are shown in figure 4. The important concept here is that as the concentration of the foliar spray was increased the amount of calcium translocated away from the leaf also increased in a regular manner. The maximum amount of calcium translocated was 0.3 μg in 24 hours. Steaming the petiole of otherwise uninjured leaves completely prevented export of Ca^{45} thereby indicating that movement was via the phloem.

Comparisons can be made between calcium and phosphorus translocation under similar conditions of application when using a 30 mM foliar spray. This is the highest phosphorus concentration available for comparison (9). The approximate values for calcium and phosphorus translocation, the latter applied as $NaH_2P^{32}O_4$, was 0.02 and 15 μg respectively, or a ratio of 1 : 750. The spray concentration resulting in the most efficient translocation of calcium was between 50 and 100 mM (fig 4) whereas for phosphorus it appeared to be between 10 and 30 mM (9). At the most efficient concentration of each approximately

Table I

Distribution of Ca^{45} in the Bark and Wood of the Stem Following Ether Treatment of the Leaflet Receiving the Foliar Spray.*

Section	Migration period following 8 min ether treatment					
	6 min		11 min		22 min	
	Bark	Wood	Bark	Wood	Bark	Wood
	counts per minute per section					
3 up	0	16	24	139	116	12,600
2 "	0	83	39	298	4,170	27,450
1 " **	25	102	44	394	4,540	35,750
1 down	180	597	500	1700	9,640	73,250
2 " ***	0	104	0	375	1,848	43,950
3 "	0	0	0	0	53	360
4 "	0	0	0	0	16	83
5 "	0	0	0	0	0	15

* The foliar spray consisted of 32 μc of Ca^{45} in 50 μl of 0.384 M $CaCl_2$ (pH 4).

** Node where tracer entered stem.

*** Primary leaf node in this section.

0.06 μg of Ca and 6 μg of P were translocated—a ratio of 1 : 100. This was approximately 0.04 % and 12 % of the calcium and phosphorus respectively which was applied to the leaves.

Injury to the leaf occurred with foliar sprays of 0.1 M $CaCl_2$ and above. A water-soaked appearance of the treated area characterized the injury and necrotic spots developed as the concentration reached 1.0 M. As will be shown later the magnitude of injury was insufficient to cause artifacts in movement unless undue stress was placed on the water columns within the plant.

Attempts to Increase Foliar Export: *1. Nutrient Treatments.* There was no indication that nutritional conditions involving the level of phosphorus, calcium, or sulfur, or the hydrogen ion concentration within the limits herein used, had any influence on calcium translocation away from leaves. This was investigated rather thoroughly because of an earlier report (1) that calcium became mobile in the phloem under growth conditions of low calcium, low phosphorus, and pH 4.0. This earlier result must now be attributed to some undetected injury to the plant which enabled calcium to enter the xylem and be distributed within the plant during the drying process. In 6 replications of the above conditions no plant showed any marked movement of calcium away from the leaf. It should be stressed that the radioautograms of dried plants all gave evidence of a very small amount of calcium being translocated away from the treated leaf. The amount, however, was consistent with the quantitative figures given in figure 4 for uninjured plants.

2. Foliar treatments: a. The Effects of NaCl: A foliar spray of 0.03 M $CaCl_2$ and 1.18 M NaCl resulted in injury to the leaf at the site of application. The injury was manifested by a water-soaked appearance, a partial collapse of the mesophyll, and an ultimate development of brown spots scattered over the area contacted by the spray. The leaf, however, remained turgid. The data, shown in figure 5, indicate that the injury was not severe enough to cause abnormal calcium movement during the 24-hour migration period. However, it was found that a very marked movement of calcium occurred during the drying process if the plants were left intact.

b. The effects of triiodobenzoic acid: A complete foliar treatment with 100 ppm TIBA had no stimulating effect on calcium movement during a 24-hour migration period. In fact the treatment appears to have hampered normal calcium transport. But, as above, a very pronounced movement occurred during the drying process if the plants were left intact (fig 5). The visual effects of the TIBA on the plants were a twisting of the terminal bud and adjacent tissue and a cupping of the leaves which were still expanding.

c. The effects of ether: Exposing a Ca^{45} treated leaflet to the vapors from liquid ether during the 24-hour migration period resulted in a complete collapse of the leaflet and a very pronounced Ca^{45} movement from the leaf (fig 5). No additional effect was observed if similarly treated plants were dried intact before being analyzed.

When using our refined method of exposing leaves to ether vapor it was found that a treatment period of less than 6 minutes had no effect on Ca^{45} movement and no visible effect on the leaflet. An 8 minute treatment caused about 50 % of the leaves to flag slightly and all treated leaves to lose Ca^{45} to other parts of the plant. Longer treatment caused complete collapse of the leaflet and a near maximal movement. An 8 minute treatment period proved best for

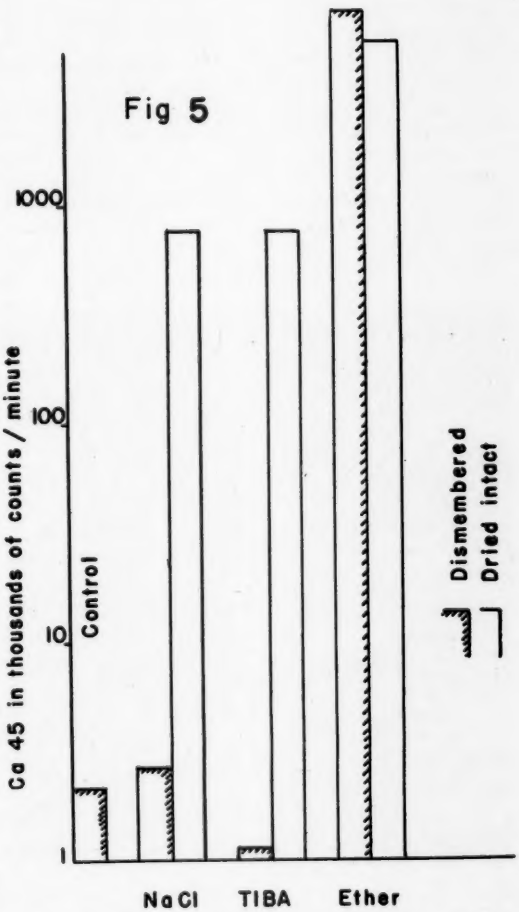


Fig. 5. The amount of foliar applied tracer calcium moving from the treated leaf to all other parts of the plant in 24 hours following several treatments and including 1 normal control. For details of treatment see text. Each member of the pair was treated similarly except that before drying one was dismembered to show the movement which had occurred during the migration period (crosshatched) from the movement which occurred during drying (plain).

the study of tissues involved in transport when the transport was induced by ether. The results are shown in table I.

An analysis of the results shows: (A) That the Ca^{45} moved from the leaf only when the controlling cells comprising the bundle sheath, or the bundle sheath extensions, around the veinlets of the leaf, were injured to the extent that they permitted the leaf to serve as a source of water and minerals to other parts of the plant. (B) That the pathway of movement from the leaf was initially the xylem. The Ca^{45} which subsequently appeared in the phloem both above and below the node of the treated leaf entered this tissue by lateral movement from the xylem. This appears to be true both because the extent of the Ca^{45} is not as great in the phloem as in the xylem and because the actual quantity of Ca^{45} in the phloem is only one-fourth to one-seventh that in the xylem. At the same time, there was little or no evidence that negative tensions developed in the phloem and were responsible for rapid calcium advancement in this tissue. (C) That the Ca^{45} descended in the xylem elements of the leaf traces to the next lower node where anastomosing of bundles occurred before it ascended to leaves higher on the stem. Only a small amount moved on downward toward the base of the stem.

Figure 6 is included to show that in a stripped stem the break between "bark" and "wood" occurs at the cambial region, effectively separating the phloem from the xylem.

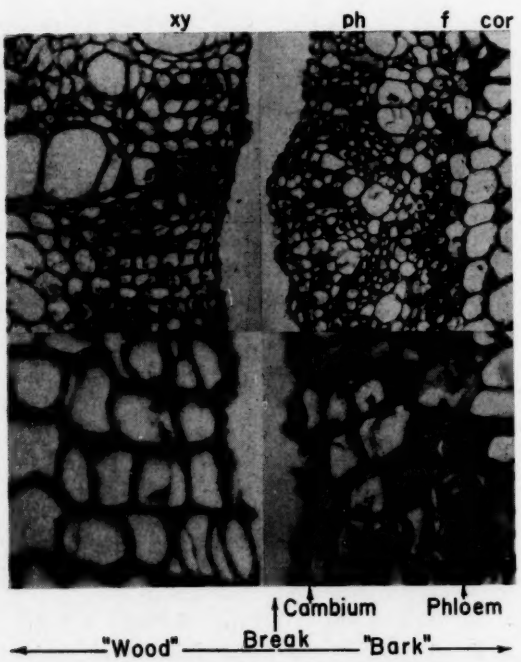


FIG. 6. Photomicrographs of a stripped bean stem showing that the break between "bark" and "wood" occurs at the cambium. Upper pair shows the break over an extended area: xy = xylem, ph = phloem, f = fibers, cor = cortex. Lower pair, at higher magnification, shows details of the break at the cambial region. Sieve plates are visible in some of the sieve elements of the phloem.

DISCUSSION

We have shown that the withdrawal of calcium from the cotyledons during the early growth of the seedling is the most striking incidence of calcium mobilization within the plant. This is in agreement with a recent report of Lausch (10). Since cotyledonary calcium is limited in amount, an external calcium source is required by the time the rapid growth of the trifoliate leaves begins. The cotyledonary calcium incorporated in the older tissues is by then relatively immobile and little is available for subsequent growth.

The calcium from the nutrient medium which enters the leaves via the transpiration stream likewise remains relatively immobile after the initial deposition. This was found to be true under a wide variety of nutrient conditions so that the traditional view that calcium is relatively immobile within the plant can be supported as a generality.

Results obtained with foliar applied Ca^{45} also show that the amount of calcium translocated from leaves is comparatively small although it is definitely measurable with radioisotope techniques. The amount has been found to compare poorly with the amount of phosphorus withdrawn. Under conditions of equal application, i.e. 30 mM, only 1 μg of calcium to approximately 750 μg of phosphorus is exported. At the most efficient concentration for exportation of each the ratio is 1 : 100. Phosphorus, in turn, has been found to be exported in far lesser quantity than sucrose under certain experimental conditions (3), the ratio being in the order of magnitude of 1 μg of phosphorus to 1 mg of sucrose. To show calcium withdrawal from foliage leaves by other means than radioactive tracing would seem very difficult as the amount involved is so small; however, Lausch (10) has recently reexamined this possibility and claims some success.

Were calcium more mobile within the plant it might be anticipated that foliar sprays could be used to alleviate certain calcium insufficiencies which develop during maturation of fruits under some environmental conditions, e.g. black end of tomatoes. This would justify a search for a calcium carrier to render it mobile, or for conditions which would increase its mobility.

To the authors, it does not seem possible to mobilize calcium by reversing polarity in the phloem as has been suggested by Bukovac et al (6) and Kessler and Moscicki (8), for when positive and negative ions enter the stem from a leaf it is known that each may move either upward or downward in the phloem. This would preclude a mechanism of movement based on electrical polarity, and polarity in any other sense

has no precise meaning, unless elaborate systems of carriers and peculiarly charged channels were invoked. Furthermore, in the present study, when calcium was induced to move from the leaf by some form of injury it definitely moved via the xylem. This is consistent with the early work of Yendo (14) and others (see Roach (12)) wherein back transport in the xylem of dyes and other markers was attained following application to cut leaves, etc., and with the work of Pallas and Crafts (11) who observed artifacts in the translocation of C^{14}-labeled 2,4-D when plants were frozen before they were dried. Perhaps this interpretation is also consistent with the results of Bukovac et al and of Kessler and Moscicki, and all are concerned with the movement of fluids in the xylem as a result of stresses which occur or develop in other parts of the plant. Only the mechanism of injury which permitted entry into the xylem differed.

Summary

The instance of greatest calcium mobilization was that from cotyledons during the early growth of root, hypocotyl and primary leaves. The growth of apical meristem and trifoliate leaves was dependent on an external source of calcium since the cotyledonary calcium was not only limited in amount but by then largely immobile.

The order of increasing calcium concentration among plant parts was roots, stems, trifoliate leaves, and primary leaves—indicating that an important factor in calcium accumulation was the total volume of the transpiration stream received. Also important was the meagre loss following the initial deposition, prohibiting reutilization in growth and metabolism elsewhere.

The amount of foliar-applied calcium exported from a leaf was small, approximately only 1/100 that of phosphorus under conditions of maximum percentage translocation. The amount exported from a leaf was related to the amount applied, but not to the nutritional conditions imposed on the plants. Calcium, then, is not immobile in the phloem; it is the volume of flow which is small.

Calcium could not be induced to move from a leaf via the phloem in extra-ordinary quantities, but movement via the xylem occurred following treatment of the leaf with the vapor of diethyl ether. Applying NaCl with the foliar spray, or treating the plant with triiodobenzoic acid induced calcium movement in the xylem during the dehydration of the plant.

The writers wish to acknowledge the assistance of Dr. Harold Koontz in accumulating the data for figure 4.

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FIXATION OF CARBON DIOXIDE IN PARTICULATE PREPARATIONS FROM BARLEY ROOTS [1]

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Non-photosynthetic fixation of CO_2 has been shown to occur in a wide range of tissues and more recently investigations have been focussed on the mechanisms of the carboxylation reactions involved. Mazelis and Vennesland (19) have summarized the 3 different carbon fixing processes as a) the malic enzyme reaction (20), b) the phosphoenolpyruvate carboxykinase (or pepcarboxykinase) reaction (29, 30), and c) the phosphoenolpyruvate carboxylase (or pepcarboxylase) reaction (3). The widespread occurrence of these 3 enzymes in higher plants has been shown (19, 31), and their possible physiological functions have been discussed (19, 27, 33).

Fixation of CO_2 by roots has been reported by various workers (7, 11, 12, 18, 21), however few investigations have been made on the carboxylation reactions involved. As ion absorption influences the level of both fixation and labelled malate formation (12, 22), and as it has been suggested that the mitochondria may act as ion carriers in ion absorption (23), it was of interest to determine whether the mitochondria may act as a site for CO_2 fixation in roots and also to investigate the mechanism whereby fixation is accomplished. Our findings have been reported briefly elsewhere (8, 35), and the effect of salts on the CO_2 fixation in the particulate preparations is reported in another paper (36). Independent evidence of pepcarboxylase activity in extracts of Snap bean roots has also been reported (10).

MATERIAL AND METHODS

The particulate preparations were isolated from excised roots of 6-day-old barley seedlings. Initially the variety California Mariot was used; in later experiments Tennessee Winter. The seedlings were grown in a dilute nutrient solution in the dark (13). The excised roots were centrifuged for 5 minutes at 65 × G to remove the excess water and then chilled at 2.5° C for at least 1 hour. Initially the excised roots were ground with sand in a mortar, but this was unsatisfactory owing to the very low CO_2 fixation activity of the preparations (0.1 % incorporation of the total carbon-14 added). The most active preparations were obtained by blending roots in a Waring blendor for 1 minute in a root to solution ratio of 1 : 1 chilled medium of 0.5 M sucrose, 0.001 M versene, and 0.05 *M* potassium phosphate at pH 7.6 to 8.0. In some experiments particles were isolated in 0.5 M sucrose solution buffered with 0.05 M TRIS [3]; but these preparations had less than 30 % normal fixation activity. The homogenate was filtered through 4 layers of cheesecloth, and the filtrate was centrifuged at −5° C in a Servall centrifuge at 1,200 × G for 10 minutes to give the low speed residue consisting of cell nuclei, cell walls, larger particles and isolated cells. The supernatant was centrifuged at 16,000 × G for 20 minutes. The pellet obtained was suspended in 0.4 M sucrose solution and recentrifuged at the same speed for another 10 minutes to give the high speed residue. The pellet was ground with a glass homogenizer, and 0.4 M sucrose solution was added to make a final particulate preparation containing 3 parts solution to 1 part pellet.

The labelled potassium bicarbonate solution was prepared by liberating CO_2 from carbon-14 labelled barium carbonate with acid and absorbing the labelled CO_2 in KOH solution. The specific activity of the $KHC^{14}O_3$ solution was 2.5 × 10^5 cts per sec per micromole. The tricyclohexylamine salt of PEP was obtained from the California Foundation for Biochemical Research; DPNH was generously donated by Dr. E. E. Conn, TPNH by Dr. R. Whatley, and phosphoserine by Dr. J. A. Gladner of the National Institute of Health, Bethesda.

The particles were exposed to $C^{14}O_2$ in Stanley manometer flasks (26) at 25° C. After an equilibration period the labelled bicarbonate was added to the reaction medium from the fixed side arm. At the end of the exposure period the reaction was terminated by the addition of 0.5 ml 2 *N* H_2SO_4 from 1 of the movable side arms, and the other side arm containing 0.5 ml 5 *N* KOH was opened to allow the absorption of excess $C^{14}O_2$ for a period of 15 minutes. Any residual labelled CO_2 was displaced by bubbling inert CO_2 through the reaction medium for an additional 15 minutes. In experiments where DPNH was present in the reaction medium, the DPNH was added after the equilibration period from the side arm

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[3] The following abbreviations will be used: TRIS, trishydroxymethylaminomethane; PEP, phosphoenolpyruvic acid; DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; IDP, inosine diphosphate; ITP, inosine triphosphate; GDP, guanosine diphosphate; UDP, uridine diphosphate.

which normally contained KOH; in these experiments the excess $C^{14}O_2$ was removed solely by bubbling with inert CO_2. Blank experiments, using heat-inactivated particulate preparations, showed that the removal of the labelled CO_2 was complete by this method.

The contents of the flask were poured into centrifuge tubes and centrifuged for 5 minutes in a clinical centrifuge; no attempt was made to obtain a quantitative recovery. Aliquots of the supernatant and the residue fractions were plated out on copper discs and the activities of the samples were determined using a windowless flow counter. Knowing the dilution factors the total amount of $C^{14}O_2$ fixation in the preparation was estimated. In all cases, the layer of the plated sample was so thin that self absorption was negligible when calculated from the data of Libby (18).

Carbon-14 activity in the organic acids was determined by ether extraction of aliquots of the supernatant fraction followed by silica gel chromatography after the method of Bulen et al (6). The 2,4-diphenylhydrazones of the keto acids were prepared according to the method of Virtanen et al (32) from aliquots of the supernatant fraction and the carbon-14 activity in the hydrazones was measured. Identification of the labelled keto acids was made by 1-dimensional paper chromatography of the hydrazones with hydrazones prepared from known standard keto acids using 0.2 M phosphate buffer at pH 8.0 followed by radioautography. Radioautographs were also prepared from aliquots of the supernatant fractions; initially 2-dimensional paper chromatograms were run using phenol: water and butanol: proprionic acid: water as the 2 solvents as recommended by Benson et al (5). In later experiments 1-dimensional chromatograms were made using the solvent system isopropanol: ammonia: water (70: 10: 20) and in this system the solvent was allowed to traverse the paper and drip from the end for a period of 3 days. This solvent not only gave a better separation of the phosphorylated compounds, but also the spots were unaffected by the presence of the salts in the reaction medium. Isopropanol: ammonia: water system had been used for some of the hydrazones. Protein hydrolysis of the residue fraction was carried out using either 2 *N* NaOH or 6 *N* HCl in sealed tubes at 117° C for a period of 12 hours, and aliquots of the hydrolysates were run on 2-dimensional paper chromatograms.

TABLE I

OXIDATION OF ORGANIC ACIDS BY BARLEY ROOT PARTICLES

SUBSTRATE ADDED	O_2 UPTAKE μl/MG N/HR
None	0
α-ketoglutarate	53
Succinate	53
Citrate	34
Malate	56
Glutamate	45
Pyruvate trace of malate	63

Reaction mixture: 1 ml particles containing 1 to 2 mg nitrogen, 60 μM substrate, 120 μM sucrose, 30 μM glucose, 9 μM AMP, 9 μM magnesium chloride, 30 μM sodium fluoride, 45 μM phosphate buffer at pH 7.4, 1 mg cytochrome c and 0.10 mg DPN in a total volume of 3 ml.

Experimental conditions: Temperature 25° C. O_2 uptake measured manometrically.

TABLE II

$C^{14}O_2$ FIXATION IN BARLEY ROOT PARTICLES IN THE PRESENCE OF PHOSPHOENOLPYRUVATE AND PYRUVATE *

A. DISTRIBUTION IN FRACTIONS

SUBSTRATE	FRACTIONS		
	SUPERNATANT	RESIDUE	TOTAL
1. Pyruvate (sodium salt)	1.1	1.0	2.1
2. Phosphoenolpyruvate (cyclohexamine salt)	12.0	2.6	14.6

B. EFFECT OF pH

SUBSTRATE	pH		
	4.8	5.6	7.4
1. Pyruvate (sodium salt)	1.4	1.5	0.3
2. Phosphoenolpyruvate (cyclohexamine salt)	2.0	2.6	12.0

* Activity as % of total carbon-14 added.

Reaction mixture: 1 ml particles containing 1.30 mg nitrogen, 60 μM substrate, 4.5 μM magnesium chloride, 0.6 μM manganese chloride, 90 μM phosphate buffer at 1. pH 5.6, 2. pH 7.4, and 1 μM potassium bicarbonate-^{14}C with 2.5 $\times$ 10^5 counts/sec carbon-14 activity in a total volume of 3.3 ml. In addition: 1. 9 μM ATP; 2. 9 μM AMP.

Experimental conditions: Temperature 25° C; exposure period 1 hr.; reaction terminated by 0.5 ml 2 *N* sulphuric acid; excess $C^{14}O_2$ removed by bubbling with inert CO_2 for 15 minutes.

RESULTS

Initial experiments were carried out to determine the ability of the particulate preparations to utilize various organic acids as substrates. The rates of O_2 uptake in the presence of added organic acids are given in table I. The results indicated that the particulate preparations were able to utilize these organic acids as substrates and that they probably possessed the enzymes necessary for the functioning of the Krebs tricarboxylic acid cycle.

$C^{14}O_2$ fixation was studied in the presence of either added PEP or added pyruvate and the results are shown in table II. The presence of PEP greatly increased the level of CO_2 fixation as compared to the fixation with pyruvate, and this increase was mainly in the supernatant fraction. The effect of

added pyruvate to the medium as compared to no added substrate was variable, in some cases giving no increase in the level of CO_2 fixation and in other cases a slight stimulation. Throughout the present investigation either pyruvate or PEP was added to the reaction medium, and these conditions for CO_2 fixation were termed the pyruvate and PEP systems respectively.

The effect of pH was studied in the 2 systems and CO_2 fixation was measured at pH values 4.8, 5.6 and 7.4 (table II). The results showed that the optimum pH requirements for the 2 systems differed; in the presence of pyruvate the highest activity was at 5.6, whereas in the presence of PEP it was 7.4. The rate of fixation by the 2 systems was measured and it was found that fixation was practically completed in 20 minutes in the supernatant in the PEP system, while in the presence of pyruvate the fixation continued to increase with time over a period of 1 hour (table III). Both systems were heat-labile and no CO_2 fixation took place in heat inactivated preparations. The PEP system in the particulate preparations retained its activity during storage and could be kept in a refrigerator for as long as a month without much loss of activity, whereas the pyruvate system lost its activity overnight.

Radioautographs of the pyruvate system supernatant showed activity in asparagine, aspartate and glutamate with traces of activity in malate. In corresponding radioautographs of the PEP system most of the activity was distributed between aspartate, glutamate, malate and phosphoserine; the distribution of activity in these compounds varied with pH with a marked increase in phosphoserine at pH 5.6 compared to pH 7.4. Hydrolysates of the residue fractions showed a wide range of amino acids and radioautographs showed labeling into most of the amino acids present. No attempt was made to identify these amino acids. Radioautographs of the 2,4-diphenylhydrazones showed that in both systems labelled oxalacetate and pyruvate were formed. A large amount of labelled oxalacetate along with trace of labelled glyoxalate appeared in the presence o PEP.

It was found that in the PEP system approximately 80 % of the fixation occurred in the hydrazones as compared to about 22 % in the pyruvate system. These results, together with the radioautograph data indicated that conditions were limiting for the further metabolism of the labelled oxalacetate. Preliminary spectrophotometric measurements of the oxidation of DPNH demonstrated that the particulate preparations contained malic dehydrogenase. The addition of DPNH to the PEP system resulted not only in an increase in the total CO_2 fixation, but also in the carbon-14 activity of the hydrazone fraction and the ether soluble fraction, in fumarate and succinate, particularly in malate. The addition of malate to the reaction medium, both in the absence or presence of DPNH, stimulated fixation. Measurements of the carbon-14 activity in the 2,4-diphenylhydrazones showed that even in the presence of added DPNH considerable activity still remained in the keto acids. Although increasing the concentration of added DPNH in the PEP + DPNH treatment resulted in a higher level of fixation in the hydrazone fraction, the presence of added malate decreased fixation. The results of these experiments are summarized in table IV.

The labelled malate was degradated to determine the distribution of the carbon-14 within the acid using a similar procedure to Jacobson (12). Trial runs showed that approximately 75 % of the malate was decarboxylated and determinations of the activity of the CO_2 produced and the remaining activity in the reaction medium in the form of pyruvate and non-decarboxylated malate indicated that the activity in malate was equally divided between 2 carboxyl groups.

The effect on CO_2 fixation of other organic acids was investigated; addition of oxalacetate (18 micromoles (μM)) to the medium greatly decreased fixation both in the presence or absence of DPNH. The presence of isocitrate (60 μM) in the PEP + DPNH system resulted in a decrease in fixation and paper chromatographic analysis of the 2,4-diphenylhydrazones of the keto acids showed an increase in the formation of labelled glyoxalate under these conditions. When the preparations were exposed to added isocitrate in the presence of TPNH, labelled glycollate was formed.

In the pyruvate system the effect of adding malate, or malate and DPNH, differed from the PEP system in that the CO_2 fixation was reduced rather than increased (see table V). In order to stimulate malic enzyme activity TPNH was added, however in both the PEP system and the pyruvate system the presence of TPNH resulted in a reduction in the CO_2 fixation (table V). The level of fixation in the pyruvate system being so low the small reduction caused by TPNH may not be significant. Fixation in the pyruvate system in this experiment was carried out

TABLE III

$C^{14}O_2$ FIXATION IN BARLEY ROOT PARTICLES IN RELATION TO TIME

TIME (MN)	CARBON-14 ACTIVITY (COUNTS/SEC).			
	PEP SYSTEM		PYRUVATE SYSTEM	
	SUPERNATANT	RESIDUE	SUPERNATANT	RESIDUE
2	1,830	34	189	14
4	3,580	156	301	32
6	5,230	184	343	37
8	6,730	266	437	64
10	7,850	318	371	51
20	12,350	790	437	103
30	11,600	1,044	579	200
60	15,300	1,420	1,140	300

Reaction mixture: 1 ml particles containing 1.10 mg nitrogen, otherwise as in table II.

Experimental conditions: See table II, except for different exposure periods.

TABLE IV

$C^{14}O_2$ FIXATION IN BARLEY ROOT PARTICLES BY THE PEP SYSTEM IN THE PRESENCE OF ADDED DPNH *

TREATMENT	PEP	PEP + MALATE	PEP + DPNH	PEP + × 2 DPNH	PEP + DPNH + MALATE	PEP + × 2 DPNH + MALATE
Total fixation	13.08	28.27	28.27	29.76	30.77	24.58
Total ether extract	0.86	1.64	6.04	7.73	12.99	10.56
Fraction						
Acetate	0	0.01	0.06	0.06	0.12	0.07
Pyruvate	0	0.01	0.03	0.02	0.03	0.03
Fumarate	0.06	0.14	0.88	1.22	2.16	1.58
Succinate	0.09	0.22	0.33	0.13	0.25	0.20
Pyrrolidone carboxylate	0.31	0.38	0.30	0.21	0.34	0.23
Oxalate	0.04	0.08	0.05	0.11	0.10	0.30
Malate	0.29	0.66	4.13	5.81	9.69	7.74
Isocitrate	0.03	0.10	0.18	0.11	0.25	0.28
Citrate	0.03	0.06	0.06	0.05	0.04	0.10
Tartrate	0.01	0.02	0.02	0.01	0.01	0.03
Fixation in ether extract expressed as % total carbon-14 fixed.	6.6	5.8	21.4	26.0	42.2	42.5
Hydrazones	10.98	19.90	15.09	11.80	14.10	11.33
Fixation in hydrazones expressed as % total carbon-14 fixed.	79.6	70.9	53.2	39.6	45.8	46.1

* Activity as % of total carbon-14 added.

Reaction mixture: 1 ml particles containing 1.23 mg nitrogen, 1 μM PEP (cyclohexamine salt), 3 μM magnesium chloride, 1.2 μM manganese chloride, 9 μM AMP, 90 μM phosphate buffer at pH 7.4 and 1 μM potassium bicarbonate-^{14}C with 2.5 × 10^5 counts/sec carbon-14 activity in a total volume of 4.3 ml. 1.6 μM DPNH, 3.2 μM DPNH and 18 μM malate added as indicated.

Experimental conditions: See table II.

TABLE V

$C^{14}O_2$ FIXATION IN BARLEY ROOT PARTICLES BY THE PEP AND PYRUVATE SYSTEMS IN THE PRESENCE OF ADDED DPNH, MALATE AND TPNH *

	TREATMENT	FRACTION			
		SUPERNATANT	RESIDUE	TOTAL	ETHER EXTRACT
A.	Pyruvate	0.27	0.10	0.37	0 20
	Pyruvate + malate	0.25	0.08	0.33	0.24
	Pyruvate + malate + DPNH	0.23	0.07	0.30	0.21
B.	PEP	47.00	0.29	47.30	7.10
	PEP + malate	62.10	0.25	62.40	6.30
	PEP + malate + DPNH	72.90	0.50	73.40	32.70
C.	PEP	39.16	0.56	39.72	2.21
	PEP + TPNH	32.83	0.69	33.52	2.45
	PEP + TPNH + malate	26.01	0.55	26.56	4.99
D.	Pyruvate	0.73	0.14	0.87	...
	Pyruvate + TPNH	0.62	0.12	0.74	...

Reaction mixture: A. 1 ml particles containing 1.30 mg nitrogen, 60 μM sodium pyruvate, 3 μM magnesium chloride, 1.2 μM manganese chloride, 9 μM ATP, 90 μM phosphate buffer at pH 5.6 and 1 μM potassium bicarbonate-^{14}C with 2.5 × 10^5 counts/sec carbon-14 activity in a total volume of 4.5 ml. 1.6 μM DPNH and 18μM malate added as indicated.

B. 1 ml particles containing 1.41 mg nitrogen, otherwise as in table IV.

C. 1 ml particles containing 1.01 mg nitrogen. 1.9 μM of TPNH added as indicated, otherwise as in table IV.

D. 1 ml particles containing 1.56 mg nitrogen, in a total volume of 3.9 ml. 1.9 μM of TPN added as indicated, otherwise as in A.

Experimental conditions: See table II.

at pH 7.4 as this is the optimum pH for malic enzyme activity.

During the course of the investigation a study of the cofactor requirements was made for the pyruvate system and the PEP systems, both in the presence and absence of DPNH. The results of these experiments involving various cofactors are summarized in table VI. The presence of both magnesium and manganese ions was required in all these systems. The nucleotide requirements differed in that AMP stimulated fixation in the PEP and PEP + DPNH systems, whereas it had no effect on the pyruvate system; ATP stimulated fixation in the pyruvate system but markedly reduced fixation in the presence of PEP. The addition of other nucleotides—ADP, ITP, IDP, GDP and UDP—in place of AMP all resulted in a lower level of CO_2 fixation in the PEP system. The carboxylation of PEP was partially inhibited in the presence of 0.05 μM *p*-chloromercuri-benzoate and completely inhibited with 0.. μM of the inhibitor. The use of TRIS as a buffe in the reaction medium resulted in a marked reduction in the level of fixation in both the PEP in th absence of DPNH and pyruvate systems; in the pyruvate system the level of fixation in TRIS was compared with that using phosphate buffer at pH 7.4 However, the presence of TRIS instead of phosphat in the PEP + DPNH system apparently increase fixation. The effect of flushing the reaction mediun for 15 minutes with cylinder nitrogen to produce low O_2 tensions reduced the level of CO_2 fixation in both the PEP and pyruvate systems.

TABLE VI

COFACTOR REQUIREMENTS IN $C^{14}O_2$ FIXATION IN BARLEY ROOT PARTICLES

MEDIUM	SYSTEM		
	PEP	PEP + DPNH	PYRUVATE
	Activity as % fixation in complete medium		
Complete	100	100	100
$-MnCl_2$	72	35	96
$-MgCl_2$	89	7	88
−AMP	84	86	100
−AMP + ATP	37	60	118
−AMP + ADP	51	55	...
−AMP + ITP	16	59	...
−AMP + IDP	...	95	...
−AMP + GDP	81	73	...
−AMP + UDP	81	79	...
$+PCMB\ 10^{-4}$ M	88	...	...
$+PCMB\ 10^{-3}$ M	0	...	...
$-PO_4$ buffer + TRIS Cl_2	54	120	67
Complete N_2 gas phase	51	...	64

Reaction mixture: Complete medium; 1 ml particles containing 1 to 2 mg nitrogen, 3 μM magnesium chloride, 1.2 μM manganese chloride, 9 μM AMP, 90 μM phosphate buffer at pH 7.4 (PEP and PEP + DPNH systems) and at pH 5.6 (Pyruvate system), and 1 μM potassium bicarbonate-^{14}C with 2.5×10^5 counts/sec carbon-14 activity in a total volume of 4.5 ml. Also 1 μM PEP (cyclohexamine salt) in PEP and PEP + DPNH systems. 1.6 μM DPNH in PEP + DPNH system. 60 μM sodium pyruvate in pyruvate systems. Further additions as indicated: 9 μM ATP, 9 μM ADP, 9 μM ITP, 9 μM IDP, 9 μM GDP, 9 μM UDP, 0.05 μM *p*-chloromercuribenzoate (PCMB), 0.5 μM PCMB, 30 μM TRIS chloride at pH 7.4, nitrogen gas phase-flask containing reaction medium flushed with cylinder nitrogen for 15 minutes before exposure to $C^{14}O_2$.

Experimental conditions: See table II.

DISCUSSION

The results demonstrated that the particulate preparations from barley roots were capable of CO_2 fixation; preliminary experiments with other fractions of the root extract, namely the low speed residue and the high speed supernatant (see Methods), also gave $C^{14}O_2$ fixation, so that it appeared unlikely that the carboxylation reactions were confined to any 1 fraction of the root extract. However as no steps were taken to purify the fractions, and no examination of the fractions was made, this point has not been conclusively demonstrated.

The results indicated that there were at least 2 carboxylating systems responsible for CO_2 fixation in the preparations, and that pepcarboxylase activity could be extremely high. Similar experiments with lupine root particles (unpublished data) gave the same pattern, with low fixation in the presence of pyruvate and a marked increase in fixation in the presence of PEP. The results for the barley particulate preparations showed that the amount of fixation varied considerably with different preparations from about 12 % to over 70 % of the total carbon-14 added. Presumably these differences were due to slight variations in the preparation of the material. It was also observed that the amount of fixation depended on both the concentration of labelled bicarbonate and on the amount of particles present; fixation increasing proportionately with higher concentrations of either bicarbonate or particles. For convenience the quantity of particles used in each experiment was expressed in terms of the nitrogen content.

The fixation of CO_2 in the pyruvate system was probably similar to the low levels of fixation found by others (3, 10) in the presence of pyruvate, although their exposure times were considerably shorter, and in the present investigation the fixation in the pyruvate system continued to increase with time over a period of 1 hour. The higher activity at pH 5.6 and the decrease of fixation in the presence of added TPNH indicated that this might not be the malic enzyme reaction. No appreciable quantity of labelled malate was formed even in the presence of added DPNH in the pyruvate system. The relatively high incorporation of the fixed carbon-14 activity by this

system into the amino acids, obtained from both the supernatant and the hydrolysate of the residue fraction, indicated that the labelled compounds formed in the presence of pyruvate underwent transamination and were utilized in protein synthesis.

The CO_2 fixation in the PEP system was essentially similar to the pepcarboxylase reaction (3) apart from the presence of AMP as a cofactor. The addition of other phosphate nucleotides ADP (19, 27), IDP (29), GDP (19) and UDP (29) all decreased fixation; this reduction may have been due to either pyruvic kinase activity, which would result in the breakdown of PEP to pyruvate, or pepcarboxykinase activity, which would result in the reversible carboxylation of PEP. We have been unable to offer any satisfactory explanation for the stimulation due to the presence of AMP. Evdence for pepcarboxykinase activity was found in small amounts of labelled PEP on the chromatograms. This could be derived by decarboxylation of α carboxyl labelled oxalacetate. Labelling of oxalacetate by pepcarboxylase has been shown to be almost entirely into the β carboxyl (3, 34), however our degradative studies of labelled malate showed equal labelling of the α and β carboxyl groups. A similar distribution of carbon-14 activity in malate has been found in other investigations with root material (2, 12). Furthermore, our preliminary experiments indicated an abundant labelling of the α position as well as the β position in oxalacetate.

Labelled glyoxalate formation was stimulated in the presence of added isocitrate indicating isocitritase activity (25); the addition of isocitrate to the medium resulted in the increased carbon-14 activity in the glyoxalate. The further addition of TPNH to this system resulted in the reduction of the labelled glyoxalate to give labelled glycollate (14). These results indicated that in our preparations there was the possible functioning of the glyoxalate bypass (16) and that α labelled malate might be formed by the action of malate synthetase (15). Further experiments are necessary to determine the importance of these findings.

The apparent inorganic phosphate requirement in both the PEP and pyruvate systems has also been observed in pepcarboxylase activity in wheat germ (27) although they were unable to find any exchange between inorganic phosphate and the phosphate moiety of PEP; in our experiments the use of TRIS as a buffer stimulated fixation in the presence of DPNH contrasting with the experiments without added DPNH. We have not investigated the mechanism of the formation of labelled phosphoserine in our preparations apart from observing its occurrence during fixation by the PEP system. No labelled glyceric acid (9, 24) was observed on our chromatograms, and spraying with ninhydrin showed that the carbon-14 activity was present in the amino carboxyl group of the phosphoserine.

Inhibition of pepcarboxylase activity by *p*-chloromercuribenzoate has also been reported for wheat germ preparations (27), and the reduction in CO_2 fixation under low O_2 tensions (28, 34) indicated that fixation in both the pyruvate and PEP systems depended on the normal functioning of the Krebs tricarboxylic acid cycle.

In conclusion our results have indicated that there are several carboxylating systems present in the particulate preparations of barley roots. The occurrence of an active pepcarboxylase system in roots provides 1 mechanism whereby roots may utilize both the endogenous CO_2 and that present in the soil in organic acid synthesis. The irreversible formation of oxalacetate takes place at low concentrations of CO_2 in contrast to the pepcarboxykinase and malic enzyme reactions which require much higher levels of CO_2 (28). The reduction of oxalacetate by malic dehydrogenase would result in an accumulation of malate, which has been shown to contain most of the carbon-14 activity in fixation studies with intact roots. The possible functioning of the glyoxalate by-pass would provide not only a means for malate formation under low O_2 tensions, but also a pathway for the interconversion of fat and carbohydrate (15). Products of fixation in the pyruvate system can undergo transamination and be synthesized into protein, whereas the conversion of oxalacetate to PEP by pepcarboxykinate can bring about the synthesis of carbohydrates from organic acids.

Summary

Investigations on CO_2 fixation showed that the particulate preparation from barley roots provided an active system for fixation of CO_2 through carboxylation of PEP. In the presence of pyruvate a low level of fixation was found. A study of the conditions and cofactor requirements was made for fixation by the 2 systems. The pepcarboxylase activity could be linked to malic dehydrogenase activity by the addition of DPNH to stimulate the formation of labelled malate. Evidence obtained from radioautographs indicated the presence of pepcarboxykinase activity and the possible functioning of the glyoxalate by-pass. The physiological significance of these reactions was briefly discussed.

We wish to express our gratitude to Dr. L. Jacobson for his helpful suggestions throughout the investigation and for his criticism of the manuscript, and to the Commonwealth Fund for a Fellowship to one of us (J.G.).

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CARBON DIOXIDE FIXATION AND SALT UPTAKE IN PARTICULATE PREPARATIONS FROM BARLEY ROOTS[1]

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Previous workers (4, 6) have shown that CO_2 fixation in excised roots is related to ion absorption since an absorption of excess cations results in an increase in both the CO_2 fixation and in the formation of labelled malic acid. The accumulation of cations and anions has been demonstrated in both animal (1, 5, 8) and plant (7) mitochondria, and in view of Robertson's hypothesis that the mitochondria may be involved in electrolyte accumulation in the plant cell, it was of interest to determine whether the CO_2 fixation by particulate preparations from barley roots (3, 9) is influenced by the absorption of salts.

MATERIALS AND METHODS

The particles were isolated from 6-day-old barley seedlings and exposed to $C^{14}O_2$ as previously described (3). Potassium was determined by means of a flame photometer. The organic acids were isolated by ether extraction followed by silica gel chromatography after the method of Bulen et al (2).

RESULTS AND DISCUSSION

Preliminary experiments were performed to investigate the potassium content of the barley root particles after exposure to potassium salt solutions; plants were grown in culture solutions containing 0.1 meq K/liter and 50 meq K/liter and the particulate preparations from these plants were analyzed for potassium. The results, given in table I, showed that the plants grown in the higher concentration of potassium yielded particles containing a higher level of potassium.

Particulate preparations were incubated with potassium phosphate for 45 minutes, in each case duplicate experiments were carried out with heat-inactivated particles as controls. The samples were then centrifuged for 15 minutes at 16,000 × G at −5° C, the supernatant was decanted and the residue pellet was quickly washed with 0.5 M sucrose before being transferred with distilled water to a dish in preparation for potassium analysis. The data in table I showed that particles prepared from plants grown in a culture solution containing a low concentration of potassium took up more potassium during exposure to potassium phosphate than those particles prepared from plants grown in a culture solution containing 50 meq K/liter; moreover, in almost every case, the level of potassium was higher in the heat-inactivated particles than in the active particles. As the potassium content of both active and heat-inactivated particles was proportional to the concentration of the external medium, the results suggested that there was surface adsorption of the potassium ions, and the

[1] January 15, 1959.

[2] Temporary address.

TABLE I

POTASSIUM CONTENT OF BARLEY ROOT PARTICLES

EXP. NO.	SOURCE OF PARTICLES	K (M × 10^{-4}) IN REACTION MEDIUM	POTASSIUM μM/MG N		
			INITIAL	INCUBATED	
				ACTIVE	HEAT-INACTIVATED
				No washing	
1.	A	800	8	120	140
		8	8	9	8
	B	800	33	100	120
		8	33	20	14
2.	A	1000	7	190	120
	B	1000	29	120	180
3.	A	500	14	160	110
	B	500	26	110	140
				With washing	
4	A	2000	14	22	8

Reaction medium: Exp. 1. Potassium phosphate, pH 5.6 as shown, 120 micromoles (μM) sucrose, 1 μM PEP (cyclohexylamine salt) in a total volume of 3 ml. Exp. 2, 3. Potassium phosphate, pH 5.6 as shown, 500 μM sucrose, 3 μM PEP (cyclohexylamine salt), 90 μM glucose in a total volume of 10 ml. Exp. 4. Potassium bromide as shown, 300 μM sucrose in a total volume of 15 ml. Particles washed 3 times with 0.5 M sucrose.

Incubation time: 45 minutes at room temperature.

A Roots grown in culture solution containing 0.1 meq K/l.

B Roots grown in culture solution containing 50 meq K/l.

A and B extracted with 0.05 M TRIS and 0.5 M sucrose.

higher level of potassium in the heat-inactivated particles might be explained on the grounds of a larger surface area being available due to the breaking up of the particles in heating. To remove the adsorbed potassium, the particles were subjected to successive washing with 0.5 M sucrose. The results (table I, Exp. 4) showed that after washing, the active particles contained 3 times as much potassium as the heat-inactivated particles, indicating an active binding of the cation.

To study the effects of salts on CO_2 fixation in the particles, single salts, KBr, K_2SO_4 and $CaBr_2$, were added to the reaction medium during exposure to $C^{14}O_2$. The results are given in table II. In the pyruvate system there was some correlation between the CO_2 fixation and the cations present in the reaction medium, since the addition of potassium bromide and potassium sulphate increased the level of CO_2 fixation, and the activity was highest in the presence of

TABLE II

EFFECT OF SALTS ON $C^{14}O_2$ FIXATION IN BARLEY ROOT PARTICLES *

	System			
	PEP + DPNH**		PEP†	PYRUVATE‡
	Extraction medium			
SALT	TRIS	PO_4	PO_4	PO_4
1. None	100	100	100	100
2. KBr	126	93	74	103
3. K_2SO_4	110	84	63	108
4. K—PO_4	88	...	—	—
5. $CaBr_2$	...	...	5	43

Reaction media:

* Activity as percentage of carbon-14 activity in the absence of added salt.

** PEP + DPNH: 1 ml particles containing 1.11 mg nitrogen, 1 μM PEP (tricyclohexylamine salt), 3 μM magnesium chloride, 1.2 μM manganese chloride, 9 μM AMP, 30 μM TRIS buffer at pH 7.4, 1.6 μM DPNH, 54 μM single salt.

† PEP: 1 ml particles containing 0.74 mg nitrogen, 1 μM PEP (tricyclohexylamine salt), 4.5 μM magnesium chloride, 0.6 μM manganese chloride, 9 μM AMP, 90 μM sodium phosphate buffer at pH 7.4, 200 μM single salt.

‡ Pyruvate: 1 ml particles containing 0.89 mg nitrogen, 60 μM sodium pyruvate, 1.5 μM magnesium chloride, 0.6 μM manganese chloride, 90 μM ATP, 90 μM sodium phosphate buffer at pH 5.6, 200 μM single salt incubated with 1 μM potassium bicarbonate-^{14}C with 2.5 $\times$ 10^5 counts/sec carbon-14 activity in a total volume of 3.5 ml.

Experimental conditions: Temperature 25° C; exposure period 1 hr.; reaction terminated by 0.5 ml 2 *N* sulphuric acid; excess $C^{14}O_2$ removed by bubbling with inert CO_2 for 15 minutes. Particles from roots grown in culture solution containing 0.1 meq. K/l.

potassium sulphate. However, the stimulation of the CO_2 fixation was small compared to that found in excised roots; this was possibly due to the presence of excess cations in the particles through the use of phosphate buffer in the extraction, and also to the presence of other ions in the medium. In the case of excised roots the presence of excess cations resulted in up to 90 % of the fixed CO_2 being incorporated into malate (4). With particulate preparations we have failed to obtain any appreciable quantity of labelled malate formed by the pyruvate system, even in the presence of a wide range of cofactors. In some cases we have found a considerable amount of labelled succinate formed in the presence of potassium bromide.

In the PEP[3] system the addition of salts resulted in a reduction in the level of CO_2 fixation even in the presence of added DPNH. The only exception occurred when the particles were extracted with TRIS instead of phosphate buffer; here the addition of potassium bromide and potassium sulphate increased the amount of CO_2 fixation, but the use of TRIS as an extraction medium results in a low level of CO_2 fixation (3). The very low level of fixation in the presence of calcium bromide was probably due to the injurious effects of the calcium ions and the competition between calcium and magnesium, the magnesium ions being essential for the PEP system (3). The decreased fixation in the presence of both potassium phosphate and potassium sulphate, compared to potassium bromide, indicated that there was no correlation between the CO_2 fixation by the PEP system and the particular salt present. This was further confirmed by the results for the activity of the ether soluble fraction extracted from the reaction media to which potassium bromide and potassium phosphate had been added in the presence of PEP and DPNH. Total $C^{14}O_2$ fixation in these 2 systems expressed as a percentage of the total carbon-14 added was 11.14 and 7.52, and the ether extract activity 4.61 and 3.22 respectively. These results showed that the total CO_2 fixation and the activity in the ether fraction were lower in the presence of potassium phosphate, and that the fixation into the ether extract expressed as a percentage of the total carbon-14 fixed were 41.4 and 42.8 for the 2 systems, there being no increased formation of labelled malate in the presence of potassium phosphate.

SUMMARY

Our results showed that although potassium was taken up by the particulate preparations from barley roots, we failed to observe either an increase in the total CO_2 fixation, or an increase in the formation of labelled malate, under conditions which induce excess cation uptake in excised barley roots.

[3] The following abbreviations will be used: PEP, phosphoenolpyruvic acid; DPNH, reduced diphosphopyridine; TRIS, trishydroxymethylaminomethane; AMP, adenosine monophosphate; ATP, adenosine diphosphate.

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EFFECT OF TISSUE AGE ON HEXOSE METABOLISM. I. AN ENZYME STUDY WITH PEA ROOT [1, 2]

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Through the use of a method involving the measurement of the rate of glucose 6-C^{14} and glucose 1-C^{14} oxidation (C_6/C_1 ratio) by plant tissues, Gibbs and Beevers (5) found that while immature tissue respired glucose either exclusively or to a large extent via the classical EMP[4] glycolytic pathway, the participation of the HMP pathway was increasingly pronounced as the tissue aged and differentiated. It is clear that the relative concentrations of the enzymes of the various pathways might determine the extent to which each participates in the metabolism of a given tissue. The purpose of this investigation was to determine whether the concentration of some enzymes of the two pathways varied with the developmental age of the plant tissue. Fructose 1,6-diphosphate aldolase, fructose 1,6-diphosphatase and 6-phosphogluconate dehydrogenase were selected as enzymes representing the two pathways. Also assayed were the enzymes catalyzing the anaerobic disappearance of ribose 5-P. The root of the pea plant was selected as the test tissue for the following reasons: 1) large numbers of this plant can be conveniently grown in hydroponic tanks; 2) root tissue in comparison to other plant organs showed a sharper decreasing C_6/C_1 ratio with successive tissue segments; and 3) previous publications from this laboratory (4, 6, 7) have demonstrated that these enzymes could be quickly and easily determined in cell-free extracts of this plant.

In this paper, attention is directed to the entire root. In a subsequent report, enzyme activity in parts of the root will be treated. A preliminary report of this investigation has appeared (2).

Materials and Methods

Growth of Plants: Pea seeds (*Pisum sativum* var. Alaska, Rogers Brothers Seed Company, Idaho Falls, Idaho) were immersed overnight in distilled water at 4° C. The following day which corresponds to day 1 in the table, they were washed for 15 minutes with a 3.5 % calcium hypochlorite solution, followed

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[4] Abbreviations used in this paper: EMP for Embden-Meyerhof-Parnas; HMP for hexose monophosphate pathway (this includes the enzymes catalyzing the formation of ribulose 5-phosphate and CO_2 from glucose 6-phosphate via 6-phosphogluconate); DPN for diphosphopyridine nucleotide; DPNH for reduced diphosphopyridine nucleotide; TPN for triphosphopyridine nucleotide; TPNH for reduced triphosphopyridine nucleotide; F-1,6-diP for fructose 1,6-diphosphate; R-5-P for ribose 5-phosphate; 6-PG for 6-phosphogluconate; G-6-P for glucose 6-phosphate; TRIS for 2-amino-2(hydroxylmethyl)-1,3-propanediol.

by a distilled water soaking. They were placed in stainless steel trays between moist towels. On the 5th day, the seedlings were of sufficient size to be transferred to the hydroponic tank containing the nutrient solution of Hoagland and Snyder (10). The temperature in the greenhouse was kept at 18 to 22° C. On occasion, the lack of sunlight became a growth factor (experimental period was February 13 to March 15). The few plants which became etiolated were discarded.

By day 7, when the 1st sample was taken, a short somewhat thick tap root had developed. Two sets of compound leaves and rudimentary secondary roots had appeared by the 12th day. On day 16, when the 4th sample was taken, there were 5 compound leaves. Flower primordia, 8 compound leaves and a mat of secondary roots had appeared by the 20th day. When the last samples were taken, 12 compound leaves had been formed.

Enzyme Preparations: Pea root acetone powder was prepared by homogenizing for 1 minute the thoroughly washed roots with 400 ml of acetone at −10° C in Waring blendors and filtering with suction. The dry powder was reblended with another 200 ml of acetone. When dry, the powder was screened through a plastic no. 20 sieve to remove course fibers. The powder was stored at −18° C in a desiccator containing P_2O_5. The enzyme extract was made the following day by grinding 200 mg of acetone powder with 15 ml of 0.1 M TRIS buffer, pH 8.5, at 4° C for 2 hours with a mechanical mortar. After removal of most of the insoluble material by centrifugation, the supernatant fluid was filtered through a plug of glass-wool. The volume was approximately 10 ml. Pea seed acetone powder was prepared by homogenizing 50 g of seeds which had been immersed in water for 16 hours at 4° C with 400 ml of acetone at −10° C in a Waring blendor and then treated in the same manner as the roots.

Reagents and Assays: Unless otherwise specified, the methods and materials employed for protein assay, aldolase, 6-PG dehydrogenase, R-5-P and F-1,6-diP phosphatase were as previously described (4, 6). Further reduction of TPN by the products of 6-PG metabolism is not significant since R-5-P does not bring about the formation of reduced TPN under the same conditions.

Table I

Characteristics of Plants

Day No.	No. of plants sampled	FW* per plant g	FW root** per plant g	Length top† mm	Length root mm
7	150	0.58	0 06	·10	35
10	100	0.82	0.13	30	70
13	61	1.25	0.34	50	100
16	36	2.00	0.54	150	170
20	30	2.50	0.80	230	220
24	31	3.40	0.95	370	300
29	31	4.40	1.02	500	300

* FW = fresh weight

** The root is defined here as the distance from the cotyledon to the root tip.

† Distance from cotyledon to highest point of plant.

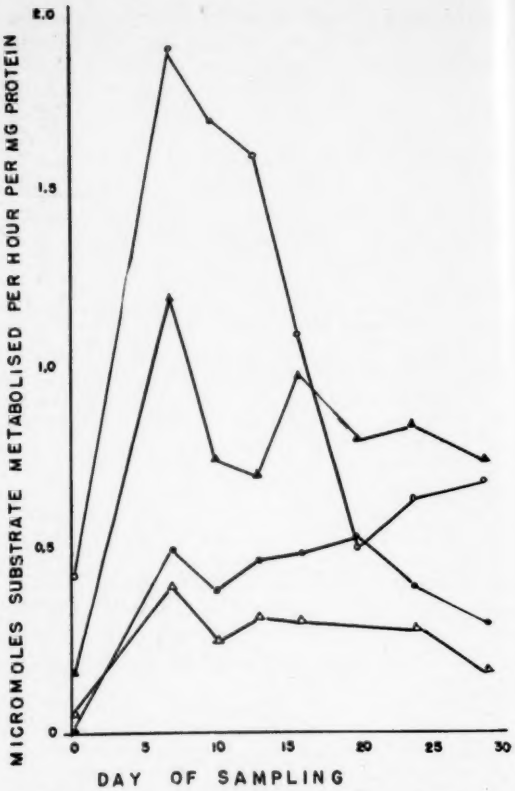

Fig. 1. ○ Micromoles of F-1,6-diP metabolized per hour per mg of protein. ● Micromoles of R-5-P metabolized per hour per mg of protein into compounds which do not yield an orcinol reaction (see ref (5)). △ Micromoles of inorganic phosphate formed per hour per mg of protein during incubation with F-1,6-diP. ▲ Micromoles of 6-PG metabolized per hour per mg of protein.

Results and Discussion

Some of the pertinent data concerning the sampling and growth of the plants are recorded in table I. They indicate that in spite of the weather conditions prevailing in the area during the winter months, growth and development of this particular crop of peas was fairly normal.

In discussing the data of figure 1, the following assumptions have been made: 1) aldolase activity represents the metabolism of G-6-P via the EMP pathway while 6-PG dehydrogenase activity reflects the entry of G-6-P into the HMP pathway; 2) the curve

designated R-5-P metabolism represents the rate at which pentose phosphate is converted anaerobically to hexose monophosphates and triose phosphates via the transketolase-transaldolase sequence of reactions; 3) the curve designated fructose-1,6-diphosphatase represents the rate of conversion of F-1,6-diP to fructose 6-phosphate; 4) a change in enzyme activity is due to a change in enzyme concentration and is not due to inhibitors or accelerators; and 5) the enzyme isolation procedures are representative for each sampling period.

The highest ratio between aldolase and the other 3 systems tested was found in the seed sample (fig 1). This would suggest that the most active mechanism for carbohydrate metabolism in this tissue is probably the EMP type. That this is the case has been demonstrated convincingly by the recent observations of Hatch and Turner (9). In their pea seed extracts, no evidence could be found for an anaerobic pathway operating alternatively to the EMP pathway. This finding of Hatch and Turner is interesting in light of our observation of an active G-6-P dehydrogenase and a somewhat more active 6-PG dehydrogenase in the seed extracts. This would suggest that the mechanism regulating the entry of G-6-P into the HMP pathway in seed extracts is not the absence of the alternative pathway enzymes. The insignificant rate of R-5-P metabolism by the extracts of seeds may be another indicator of a nonfunctional HMP pathway.

Attempts to bring about in plant extracts a partial reversal of glycolysis, i.e. the conversion of pyruvate to hexose monophosphate, have generally been unsuccessful. The rate of the forward reaction is far greater than the reverse reaction. The cause of this difference has usually been thought to be a lack of adenosine triphosphate and reduced pyridine nucleotide. Another controlling mechanism may be the amount of fructose 1,6-diphosphatase which is only 10 % as active as F-1,6-diP aldolase (fig 1).

By the 7th day, when the 1st root samples were assayed, the activity of the 4 enzyme systems had changed considerably. The system catalyzing the disappearance of R-5-P had multiplied the most. Between the 7th and 13th day, aldolase activity decreased slowly followed by a sharper drop between the 13th and 16th day. In comparison, R-5-P metabolism and fructose 1,6-diphosphate activity showed little change while 6-PG dehydrogenase decreased until day 13, then remained stable.

The 2 most striking changes illustrated in this figure are: 1) the appearance of the system catalyzing the metabolism of R-5-P between the seed sample and the first root sample which may reflect the formation of a functional HMP pathway; and 2) the sharp drop in aldolase activity between the 16th and 20th day without a concomitant decrease in 6-PG dehydrogenase and R-5-P metabolism. It is interesting to speculate that flower initiation which occurred in this period may be linked to this decrease.

In tissues which possess enzymes of both pathways the unresolved question concerns the internal influence(s) which apparently determines the fate of the G-6-P. In the embryonic tissue which appears to respire hexose mainly via the EMP sequence, an internal influence may be a high TPNH/TPN ratio. Clearly the possibility exists that as root embryonic areas differentiate during growth, a low TPNH/TPN ratio combined with a high DPNH/DPN ratio could shunt G-6-P into the HMP pathway since the one oxidizing step of the EMP pathway in pea roots is DPN-specific (3). A control mechanism of this type has been fully documented in bacterial and animal extracts (1, 8). If the C_6/C_1 ratio is an indicator of carbohydrate dissimilation, then the data presented in this paper are consistent with the concept that while the EMP pathway plays a dominant role in hexose metabolism in embryonic tissue, the HMP pathway makes an increasing contribution as aging occurs. This would further imply that enzyme concentration does, indeed influence the carbohydrate metabolism of at least part of the maturing plant. Turner and Turner (11) made a similar suggestion after demonstrating a linear relationship between the rate of starch formation and starch phosphorylase activity in newly developing pea seeds.

Summary

The purpose of this investigation was to determine whether the developmental age of the root of the pea plant had any effect on the enzyme concentration of fructose 1,6-diphosphate aldolase, 6-phosphogluconate dehydrogenase, fructose 1,6-diphosphatase and the enzymes catalyzing the anaerobic metabolism of ribose 5-phosphate. Extracts prepared from acetone powders of 7, 10, 13, 16, 20, 24, and 29 day old roots and from seed which had been immersed overnight in water were assayed. In the extracts of the seed tissue aldolase activity was found to be approximately 2.5 times higher than that of 6-phosphogluconate dehydrogenase while the enzymes catalyzing ribose 5-phosphate disappearance were essentially absent. When the first root sample was taken on the 7th day, the activities of aldolase, 6-phosphogluconate, ribose 5-phosphate metabolism and fructose 1,6-diphosphatase had approximately increased by factors of 5, 8, 50 and 8, respectively. In subsequent samples, both a decrease in aldolase activity and 6-phosphogluconate activity was found. However, the aldolase activity decreased significantly more so that by the 20th day, the dehydrogenase activity was approximately 50 % higher. The ribose 5-phosphate metabolism rate remained about half that of the dehydrogenase. The phosphatase rate was the lowest of the systems investigated being approximately one third that of the dehydrogenase. It was concluded that the concentration of enzymes of Embden-Meyerhof-Parnas glycolytic sequence and of the hexose monophosphate pathway may be an internal influence on determining the immediate fate of glucose 6-phosphate.

The authors would particularly like to thank Mr. Frank German and Mr. Robert Gaffga of the Biology Department of the Brookhaven National Laboratory for their assistance in the growing of the plants.

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AN AIRFLOW PLANIMETER FOR MEASURING THE AREA OF DETACHED LEAVES[1]

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Competition for light within crops and pastures has received considerable attention in the last few years. The area of leaf is the principal factor governing the ability of the plant to intercept light energy and thus the measurement of leaf area has been of direct importance in growth and competition studies. But measurement of leaf area has been a tedious process, particularly in mixed pastures where leaves of many species have to be measured. A rapid and accurate method of measuring leaf area despite differences in shape and texture is required. The apparatus described in this paper is presented as meeting these needs.

The more widely used methods of measuring leaf area were reviewed by Frear (2) and more recently by a series of papers presented at the Third Easter School in Agricultural Science, University of Nottingham (4). Briefly these methods are:

Blue-print and planimetering: The leaf is held flat by glass plates on light sensitive paper; on exposure and printing a record of the shape of leaf is obtained; the area is then measured by a planimeter. This method is presumably accurate, but it is very slow and tedious; it has the unique advantage in that a permanent record of individual leaf sizes and shapes is obtained.

[1] Received January 19, 1959.

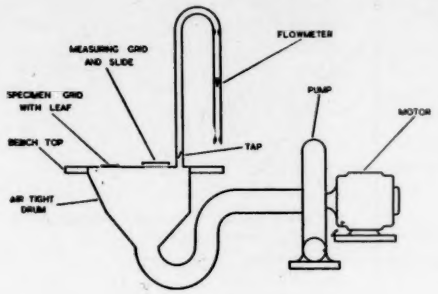
Counting squares on graph paper or glass grids: This method has been reported for the measurement of turnip leaves; with smaller leaves of pasture species and particularly with long narrow grass leaves this method would be impracticable.

Light interception: Leaves are placed between a light source and a photronic cell; the intercepted light (measured by the photronic cell) is proportional to the area of the leaves. The accuracy of this method depends on leaves being placed flat and at right angles to the column of light. Many detached pasture plant leaves will not lie flat unless held by glass plates in which case the method becomes slow and tedious.

Measured Parameters: Where the shape of the leaf is closely related to a geometric pattern the area of leaf is calculated in the same way as the particular geometric pattern; this method is not applicable to most pasture plant species. In a similar manner methods have been developed whereby the area of leaf has been empirically related to a linear measurment of some part of the leaf (1). These methods require considerable preliminary work for each species to be measured and are impracticable for most pasture samples.

Materials and Methods

Basically the apparatus (fig. 1) consists of 2 identically perforated plates mounted in the top of an airtight drum, which is connected to a constant speed



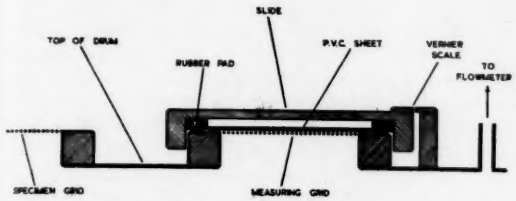


FIG. 1 (*top*). A cross sectional diagram of the air flow planimeter.

FIG. 2 (*bottom*). A cross sectional diagram showing detail of the measuring grid and part of the specimen grid of the air flow planimeter.

rotary pump. One plate, the specimen grid, is uncovered; the 2nd or measuring grid is covered by an airtight slide (fig. 4). The pressure within the drum (the datum pressure) is noted before any leaves are mounted. Leaves are then mounted on the specimen plate and are held flat by suction pressure. When all leaves are mounted the pressure is brought back to the datum pressure by opening the slide which covers the measuring grid. The area of leaf is equal to the area of the exposed portion of the measuring plate. This area is recorded by a vernier scale (figs 2 and 4) mounted on the slide.

SPECIMEN GRID AND MEASURING GRID: These grids are copper sheets punched with 0.020 in. diameter holes at 0.42 in. centers giving 625 holes per sq. in. The screens are soldered on rectangular brass frames of ½ in. square section, the inside measurement of the frames being 10 in. by 3 in. The frames are fitted over holes (10 in. × 3 in.) in a 16 gauge galvanized iron sheet which is level with the bench top. The slide is fitted over the frame of the measuring plate and the sides of the frame form part of the guide to the slide. The slide is a U shaped brass frame fitted with a polyvinyl chloride sheet, which is attached at the edges of the sheet to a thin rubber pad which is in turn attached to the frame. The center of the polyvinyl chloride sheet is free so that it can be sucked down onto the grid to form an airtight seal. (Details of this slide are shown in fig 2.)

AIRTIGHT DRUM: The drum is a rectangular tank of 20 gauge galvanized iron (48 in. long × 18 in. wide × 15 in. deep); the top of the drum is screwed to the underside of the bench. There is an 18 in. diameter hole in the bench top into which is mounted the plate carrying the specimen grid and measuring grid. A 1 in. × 1 in. cross section cast iron spacing ring is fitted between the plate and the top of the drum thus making the plate flush with the bench top.

The drum is connected to the suction pump through a 15 in. diameter hole in the bottom of the drum; this hole is reduced by a cone (6 in. deep) to 4 in. diameter and the suction pump is connected by 4-in. lead piping which passes through the laboratory wall by smooth gentle bends. The suction pump has been placed outside the laboratory wall to reduce noise. A 3 foot long perforated (½ in. diameter holes) pipe is attached to the outlet of the suction pump to reduce wind turbulence effects at the pump outlet.

In the prototype instrument a grid (½ in. square holes and 2 ½ in. deep) has been placed across the top of the cone inside the drum to smooth the turbulent air flow into the pump. This has proved to be unnecessary as very little difference due to turbulence has been noted when reading the datum pressure with the grid removed.

A quick release valve (opening 3 1/2 in. diameter) has been fitted into the drum. It has been found necessary to reduce pressure when mounting certain types of leaf e.g. the leaflets of clover tend to bunch together when mounting. Reducing the pressure avoids this difficulty.

SUCTION PUMP: The suction pump is a small forge blower which has a peak pressure of 8.5 inches of water gauge and delivers approximately 290 cubic feet of air per minute at the datum pressure of 3.75 inches of water gauge. The pump is driven by a half horsepower 3-phase squirrel cage induction motor, whose speed can be kept constant; this is essential as accuracy of measurement is dependent upon constant pressure being maintained within the apparatus.

PRESSURE OR AIR FLOW GAUGE: Many types of pressure gauges have been tried. It has been found that a standard rotameter gave accurate results. As rate of air flow through a fixed orifice is dependent on pressure difference, measurement of changes in rate of air flow will give similar results to measurement of pressure changes. The rotameter is fitted with an adjustable eye piece to set the datum air flow level.

RESULTS AND DISCUSSION

ACCURACY OF MEASUREMENT: A number of procedures were followed to test the accuracy of the apparatus.

1) Tests were made to determine whether air is drawn through the leaves, thus affecting the area

determination. Leaves of various plants were traced and cut out of thin rubber; no differences in readings were found in tests with leaves and rubber of equal area.

A 2nd test was conducted whereby leaves were blue-printed and the area determined with a planimeter; the blue-printed leaf shapes were cut out and the area of paper and that of original leaves were obtained using the air flow planimeter. No differences were recorded in these tests.

2) A test was then made to determine whether leaf shape could affect the area of leaf as recorded by this apparatus. Pieces of paper of known area were cut into many different shapes. No differences in area were recorded.

3) The apparatus was calibrated using pieces of graph paper of known area. It was found that there was a reading error of ± 0.5 sq cm. This error was constant irrespective of the total area measured; hence with a 5 sq cm sample the error was ± 10 %, but with a 100 sq cm sample the error was ± 0.5 %. (In addition a measurable correction factor has proved necessary for the prototype, as there was a constant error ranging from zero at 5 sq cm to + 7.0 sq cm at 150 sq cm.)

4) Presumably, the most accurate method of determining leaf area has been to blue-print the leaves and measure the area with a planimeter. A test was made with various samples of grass and clover leaves by blue-printing and by measuring the leaves on the air flow planimeter. The area of the clover leaves, subterranean clover (*Trifolium subterraneum* L.) var. Bacchus Marsh, was also determined using the method of Black (1) whereby the area of leaf is determined by use of the relation between midrib length and leaf area. The area of grass leaves, Wimmera rye grass (*Lolium rigidum* Gaud.) was also determined by the method of Langer (3) whereby the area of leaf is determined using the relation between length and width measurements and leaf area. The results are shown in table I.

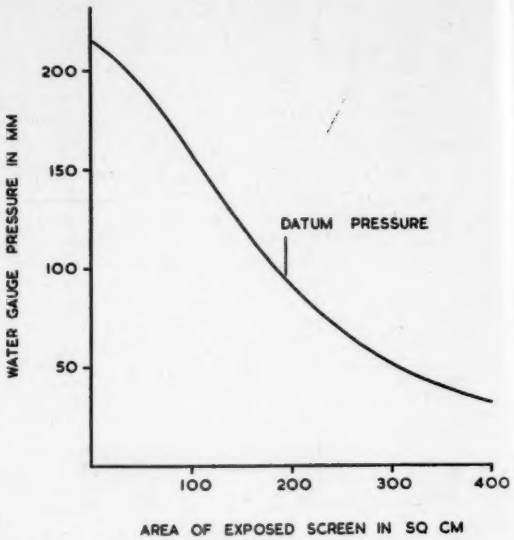


FIG. 3. Relationship between water gauge pressure and the area of exposed screen of the air flow planimeter.

While making these measurements it was found in some cases that the area of leaf contracted with time after cutting. For this reason, in the case of the clover leaves, blue-printing was done both before and after the leaves were measured on the air flow planimeter; the mean of these 2 measurements presumably gave a better account of the actual area of the leaves at the time they were on the air flow planimeter. It will be seen that there was very close agreement between blue-printing and the air flow planimeter.

5) The accuracy of measurement of this instrument depends on the magnitude of the pressure change

TABLE I

COMPARISON OF AREA MEASUREMENTS OF CLOVER AND GRASS LEAVES BY SEVERAL METHODS (SQ CM)

WIMMERA RYE GRASS				SUBTERRANEAN CLOVER VAR. BACCHUS MARSH					
No. OF LEAVES	METHOD OF MEASUREMENT			No. OF LEAVES	METHOD OF MEASUREMENT				
	LENGTH AND WIDTH (LANGER)	BLUE-PRINT	AIR FLOW PLANIMETER		MIDRIB (BLACK)	BLUEPRINT A*	BLUEPRINT B*	BLUEPRINT MEAN A & B	AIR FLOW PLANIMETER
5	35	37	36	5	49	52	52	52	52
5	34	37	36	5	58	59	59	59	59
10	72	72	74	10	87	96	94	95	94
10	67	71	71	10	73	78	74	76	75
15	113	105	103	15	185	178	174	176	173
15	125	126	127	15	151	136	134	135	136
20	143	141	142	20	192	179	167	173	174
20	153	146	148	20	201	199	191	195	194

*Blueprint A was done before and blueprint B after measuring with air flow planimeter.

per unit area. With the suction pump used in this model the pressure change with unit area is gradual and the peak pressure is approximately 8 inches of water gauge (fig 3).

Alternatively, a household vacuum cleaner can be used provided it has a high peak water gauge pressure (cf. 45 in.). It will then give a relatively high change of pressure per unit area with a reading error of only ± 0.1 sq cm. Unfortunately, however, vacuum cleaners have a very low capacity and to obtain this accuracy the specimen plate must be reduced to about 5 sq in., compared to 30 sq in. in the apparatus as described.

SPEED OF MEASUREMENT: In determining the area of samples of clover and grass leaves recorded in table I, the time required to perform each operation of the various methods used was recorded. Allowance was made for the fact that the operator was more skilled in some methods than in others. The time interval measured began when the cut herbage sample was laid on the table in the laboratory and ended when an area measurement for the sample was obtained either directly or by calculation. All measurements, whether made by 1 or 2 operators, were converted to man/seconds per leaf.

In using the blue-print and planimeter method on grass leaves it was found that area differences of up to 6 % were recorded in the measurements made by the planimeter. It proved necessary to measure each blue-print 3 times to obtain the figures shown in table I. However, the time shown is for only 1 measurement, when repeat measurements were made on particular blue-prints. The results in table II indicate the speed with which the air flow planimeter can be used.

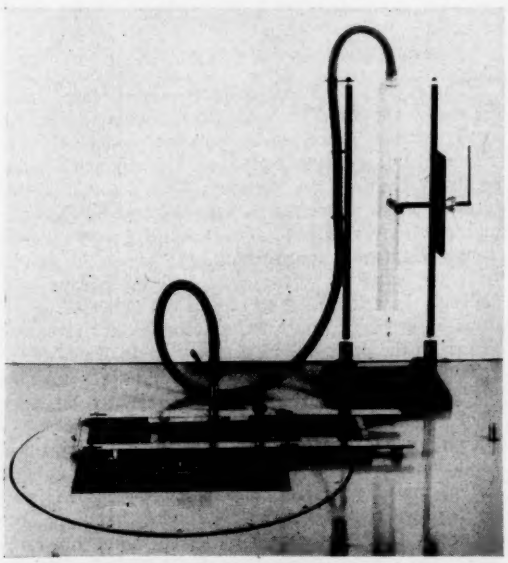


FIG. 4. The operating parts of the air flow planimeter. Clover leaves are mounted on the specimen grid and the measuring slide is partly open.

TABLE II

TIME REQUIRED FOR DETERMINATION OF LEAF AREA*

SPECIES	METHOD			
	AIR FLOW PLANIMETER	BLUE-PRINT	MIDRIB LENGTH MEASUREMENT (BLACK)	LENGTH AND WIDTH MEASUREMENT (LANGER)
Clover	11	50	17	...
Grass	12	50	...	50

* Man-seconds per leaf.

This apparatus has been used throughout the 1958 season in the agronomy laboratories of the Waite Agricultural Research Institute. It has been used for the measurement of leaf area of single plants and of samples from mixed pasture swards. It has been very satisfactory for speed and reliability and has considerable advantage in causing little fatigue for the operator. A technician can be trained in the use of the equipment in approximately 30 minutes.

SUMMARY

An air flow planimeter is described which measures the area of detached leaves accurately and quickly.

Basically the apparatus consists of 2 identically perforated plates mounted on an airtight drum which is connected to a constant speed rotary pump. The rate of air flow is noted with 1 plate covered (the measuring grid) and with the other plate open (the specimen grid); this gives a datum pressure. Leaves are mounted on the specimen grid and are held flat by suction pressure. The area of the leaves is determined by exposing part of the measuring grid to bring the air flow back to that of the datum pressure. The exposed area on the measuring grid is then equal to the area of leaves on the specimen grid. From a vernier scale on the measuring grid a direct reading of area is obtained.

The author is indebted to Mr. E. J. Leaney of the Department of Agronomy, Waite Agricultural Research Institute and to Mr. A. Palm of the Division of Soils, Commonwealth Scientific and Industrial Research Organization for continued interest and assistance in the development of this equipment.

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ANTHOCYANIN SYNTHESIS IN CORN ENDOSPERM TISSUE CULTURES. I. IDENTITY OF THE PIGMENTS AND GENERAL FACTORS[1]

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Until recently few physiological studies have been made which bear directly on the biosynthesis of anthocyanin. However, a great deal of work has been done on the chemistry and genetics of these pigments. Much of the earlier literature has been summarized by Onslow (7) and more recently Blank (1) has written an extensive review. Many of these physiological studies have been concerned with the effects of environmental factors such as light and temperature. In addition, studies have been carried out to determine the effects of feeding different sugars and sugar concentrations to seedlings of green plants (3).

Recently, Thimann and his associates (4, 16, 17, 18, 19, 20) have entered upon a new approach to the problem of anthocyanin synthesis. They chose to work with the comparatively simple green aquatic plant, *Spirodela oligorrhiza* K., which can be easily grown under known controlled conditions. With this plant, for example, Edmundson and Thimann (4) have been able to demonstrate the probable mediation of a copper-containing enzyme in anthocyanin synthesis. Thimann and Radner (20) have shown that riboflavine plays an important role in the production of these pigments. Siegelman and Hendricks (11) have determined the action spectrum for anthocyanin formation in seedlings of turnip and red cabbage. They suggest that their data strongly support the implication of a copper-containing enzyme. However, in all of those studies as well as in those by many other workers, green plants or plant parts which ordinarily photosynthesize were used and under those circumstances the effects of photosynthesis, whether direct or indirect, can hardly be discounted. Slabecka-Szweykowska (12) used callus cultures of *Vitis vinifera* stem for studying anthocyanin synthesis. These cultures do not contain chlorophyll and she was able to demonstrate conclusively that light was essential for anthocyanin synthesis in these tissue cultures. However, the production of anthocyanin by the Vitis tissue occurs only on media in which the sugar content is 3 % or greater. Thus, anthocyanin synthesis must be induced in this tissue by relatively high sugar concentrations. It would seem desirable to have a tissue devoid of chlorophyll and which synthesizes anthocyanin under ordinary circumstances as an experimental tool with which to investigate anthocyanin synthesis. Therefore, when an anthocyanin-bearing strain of corn endosperm had been isolated as a continuing tissue culture (13, 15), it was thought that this tissue might provide the ideal experimental system for these studies.

METHODS AND MATERIALS

The endosperm tissue cultures were isolated from the maize variety, 'Black Mexican Sweet', according to the methods described by Straus and LaRue (15). The nutrient medium contained White's major elements, Nitsch's trace elements (6), 0.5 % Seitz-filtered yeast extract, 2 % sucrose, and 0.9 % agar unless otherwise noted. The general growth conditions and methods for determining changes in weight were the same as those described by Straus and LaRue. In an attempt to reduce the effect of variations in the initial pigment concentration, tissue was grown in the dark. Such tissue is virtually colorless (see effects of light) and was used as a source of inocula. However, it was soon discovered that the final pigment concentrations of both light- and dark-grown tissues subjected to the same experimental conditions were usually the same with a few notable exceptions. Thereafter, explants were chosen from light-grown tissue in order to simplify handling procedures. One experiment reported here, however, was conducted with dark-grown tissue as will be pointed out later.

Because of the requirement for yeast extract, experiments which would involve extensive manipulation

[1] Received January 19, 1959.

[2] This work was begun in 1954 when the author held an American Cancer Society Post-Doctoral Research Fellowship. It has since been repeated and extended with the aid of grant G-3529 from the National Science Foundation.

of mineral nutrients and many organic compounds in the medium are severely restricted. However, work is progressing toward the development of a synthetic medium which should greatly facilitate experimentation with these cultures.

EXTRACTION AND QUANTITATIVE DETERMINATION OF THE PIGMENT: The tissue is removed from the culture vials, gently blotted with filter paper to remove excess water, weighed and placed in a flask. Two percent concentrated hydrochloric acid in 95 % ethanol (v/v) is poured over the tissue and the mixture permitted to steep for 4 hours at room temperature. At the end of this period, the tissue is removed by filtration and extracted a second time with fresh solvent. This procedure is repeated once more. The volume of the combined extracts is determined and 10 ml are used to determine the relative pigment concentration. A Klett colorimeter employing a green (no. 54) filter is used for this purpose. The Klett reading is substituted in the formula that Thimann and Edmundson (16) used for the pigments in Spirodela to give relative pigment concentration:

$$\text{P.U./g} = \text{Klett reading} \times \frac{\text{ml solvent}}{10} \times \frac{1}{\text{g tissue extracted}}$$

where P.U./g stands for pigment units per gram of tissue in 10 ml of the extract.

The first 2 extractions remove almost all of the free pigment. Steeping the tissue overnight or for several days in the 3rd solvent change removes a very slight additional amount of pigment although the tissue remains a bright pink. Heating or grinding the tissues in the solvent removes a bit more pigment, but the consequent cell disruption results in a suspension which is difficult to clarify and which interferes with colorimetric readings. Because of this, it was decided that a more or less constant error due to unextractable pigment was preferable.

To estimate the amount of pigment produced in absolute units, chromatographically purified chrysanthemin (see below) was prepared and 26.04 mg were dissolved in 100 ml 95 % ethanol containing 2 % HCl (v/v). Appropriate dilutions were made and the solution read in a Klett colorimeter with a green filter. A concentration of 0.13 mg in 10 ml gave a reading of 40. Thus 1 pigment unit is equivalent to 3.25 μg of anthocyanin. This is not entirely accurate as it does not take into account the absorption by other colored substances in the crude extract. However, the figure is probably a close approximation. Further calculations show that the pigment concentration of fresh tissue is about 0.5 %. The percent dry weight of the tissue is 7.5 %, hence anthocyanin represents about 6.5 % of the dry weight of the tissue.

CHROMATOGRAPHY: Extracts for chromatography were prepared by disintegrating the tissues in a Waring blendor with the extraction solvent mentioned above. The debris was removed by filtration and the filtrate evaporated to dryness in an air stream at room temperature. The residue was extracted with a minimum quantity of 95 % ethanol or absolute methanol and filtered. The filtrate was applied to the paper without further treatment unless otherwise noted.

The irrigating solvents used were the organic phases of the following mixtures: *n*-butanol : acetic acid : water (4 : 1 : 5 v/v; hereafter referred to as solvent 1) and *n*-butanol : 2 *N* hydrochloric acid (1 : 1 v/v; hereafter referred to as solvent 2). Whatman filter papers no. 1 and no. 3 MM were used. They were washed in *N* hydrochloric acid; then with distilled water until the washings were chloride-free; then in 0.1 % ethylenediamino tetraacetic acid (disodium salt) (w/v) and finally rinsed with a volume of distilled water equal to that needed to wash the paper free of chloride following the acid treatment. The papers were equilibrated with the aqueous phase of the solvents for 8 hours at 30° C.

RESULTS

IDENTITY OF THE PIGMENTS: Extracts prepared as previously described were spotted on Whatman no. 1 paper and chromatographed with solvent 1. Under the conditions described, 3 anthocyanins were discerned at R_f 0.3 (band 1), 0.36 (band 2), and 0.47 (band 3). The spot at R_f 0.36 represents the pigment present in greatest concentration followed in much lower concentration by the pigment at R_f 0.47. A rough estimate of the relative concentrations of these 2 pigments shows a ratio of about 9 to 1. The slowest running pigment is barely visible.

Sando et al (10) demonstrated the anthocyanin present in the husks of purple-husked corn to be chrysanthemin (cyanidin-3-monoglucoside). Coe (2) also showed that a cyanidin-3-monoglucoside was present in the aleurone layer of maize kernels of the genetic constitution, Pr. In addition, he reported the presence of callistephin (pelargonidin-3-monoglucoside) in the same tissues as well as in tissues of pr. Maize aleurone is purple when among other genetic factors, the genetic factor, Pr, is present and is red when homozygous for pr. On the assumption that these same pigments were present in the extracts of the endosperm tissue cultures, authentic chrysanthemin was prepared from extracts of flower heads of a bronze variety of chrysanthemum (7, 8, 9) and callistephin from ripe strawberries (5). Initially, an attempt was made to obtain callistephin from the deep red flowers of a Pelargonium species. However, upon chromatographing the extract, 6 poorly separated bands were found and these flowers were abandoned as a source of pigment.

The chrysanthemum and strawberry pigments were extracted in the same manner as that described for the endosperm tissues. The extracts were streaked across the shorter dimension of whole sheets (18¼ x 22¼ inches) of Whatman no. 3 MM paper and chromatographed with solvent 1. The well separated bands were cut out and eluted with 95 % eth-

anol. The solutions were concentrated and the process repeated.

To establish the identity of these pigments, the qualitative tests devised by Robinson and Robinson (8) were applied to the anthocyanins and their aglycones. The aglycones were prepared by adding an equal volume of concentrated hydrochloric acid to a solution of the pigment and heating in a boiling water bath for 15 minutes. The anthocyanidins were extracted with *n*-amyl alcohol. The Robinsons' tests established the chrysanthemum pigment as the cyanidin-3-monoglucoside; the strawberry pigment as the pelargonidin-3-monoglucoside; and the aglycones as cyanidin and pelargonidin respectively.

The endosperm pigments were purified chromatographically in the same manner as that described for the chrysanthemum and strawberry pigments. The endosperm pigment of band 2 was cochromatographed with chrysanthemin on Whatman no. 1 paper in solvents 1 and 2. The R_f values of the pigments were identical. When the 2 pigments were spotted together and permitted to advance almost to the edge of the paper (about 20 hr) in solvent 1, there was no visible separation of the pigments. The aglycone of the endosperm pigment was prepared and compared chromatographically in solvent 2 with cyanidin obtained from chrysanthemin and again the R_f values of the two were alike. The same procedures were repeated with callistephin and endosperm pigment from band 3 and again identical R_f values and non-separation of the pigments were obtained. Thus, it seems quite reasonable to conclude that the 2 endosperm pigments investigated are chrysanthemin and callistephin. The 3rd pigment is present in such minute amounts that it contributes very little to the total pigmentation and no attempt was made to establish its identity.

Absorption Spectrum: The absorption spectrum of the crude endosperm extract prepared by steeping the tissue was determined with a Beckman Model DU spectrophotometer. The combined extracts were diluted to a convenient color density and readings were taken over the range of 310 to 600 mμ. Two absorption peaks were consistently found: one at 325 mμ and another rather broad one at 530 to 540 mμ. The peak at 325 mμ is probably due to flavone pigments (12, 16). An absorption spectrum of a corn endosperm tissue culture derived from a variety of corn that is normally unpigmented did not show any peaks within the same range of wave lengths. The spectrum of a colorless tissue which arose spontaneously from the anthocyanin-producing tissue, however, showed strong absorption in the same near ultra-violet region as did the pigmented tissue, but no peaks were found at longer wave lengths (14).

The absorption spectra of the purified anthocyanins were also determined. The solvent used for this purpose was 2% hydrochloric acid in 95% ethanol (v/v). Chrysanthemin (band 2) had peaks at 272 and 540 mμ; callistephin (band 3) had peaks at 275 and 520 mμ.

Growth-pigment Curve: Comparison of individual cultures shows variations in the intensity of pigmentation from culture to culture. In preparing pools of tissue for explants, uniformly pigmented tissue was chosen as far as practicable. Newly transferred tissue has a uniformly pigmented surface, but the interior is of a lighter hue. When growth commences in a fresh explant, the new tissue is devoid of any visible anthocyanin, but as time progresses, pigment begins to appear in the older portion of the new tissue. Thus, during a period of active growth, the cultures have the general aspect of a pigmented island surrounded by a non-pigmented peripheral area. However, at the end of the growth period (25 days) all of the tissue is pigmented. Growth of the tissue is very uniform, but pigment production is rather variable. This may be due to the difficulty in providing uniform lighting under the conditions in which the cultures are grown. Because of this variability in pigment concentration, controls were run with each experiment using tissue from the same pool as that used for the experimental series. The results are therefore not corrected for initial pigment concentration. Indeed, corrections for initial pigment concentration do not seem to be necessary because when tissues grown in the dark and therefore virtually colorless, are used as inocula, the resulting cultures produce as much pigment at the end of 25 days as when inoculated from light-grown tissues.

In order to determine the time of maximum pigment concentration, 300 cultures were prepared and the wet weight and pigment content of 50 vials of tissue were determined every 5 days for a total of 30 days. The data are shown in figure 1. After several days growth, there is a general trend toward lower pigment concentration up to 20 days. This is because newly produced tissue is devoid of pigment so that the net effect is one of less pigment per unit of tissue. After 20 days, however, when growth begins to slow down, anthocyanin synthesis continues and there is then a slight net increase in pigment content. All experiments to be described hereafter were terminated on the 25th day after preparation.

Effect of pH: A series of cultures was prepared with the pH of the medium varying from 3.2 to 8.7. The media were prepared by adjusting the pH of the yeast extract solution with HCl or KOH and adding the extract to the rest of the medium. A pH of 5.0 favors pigment production in these tissues (fig 2). These results differ from those of Thimann and Edmundson (16) who found that a pH slightly above neutrality was most favorable for anthocyanin synthesis in Spirodela.

Effect of Light: Only the general effects of light were tested. Cultures grown in complete darkness produce between 180 and 300 pigment units per gram of tissue in contrast to those cultures grown in the diffuse light of the laboratory which produce between 1520 and 1600 pigment units per gram. The pigment in the dark-grown tissue may be due to a dilution of the pigment present at the outset of the

experiment due to the growth of the tissue. However, the possibility of some synthesis in the dark cannot be overlooked (16). The final increases in weight of the light- and dark-grown tissues were not significantly different. Much of the tissue produced in the dark is colorless. When such tissue is exposed to the ordinary light of the laboratory, pigment first becomes visible in about 6 hours. This evidence clearly supports Slabecka-Szweykowska's (12) view of the influence of light on anthocyanin synthesis without the complications of photosynthesis mediated by chlorophyll.

EFFECT OF SUGARS: Table I summarizes the effects of glucose, fructose, and sucrose as well as those of different concentrations of sucrose. Sucrose is the best of the 3 sugars in promoting pigment production. Glucose is least effective and fructose is intermediate in effectiveness. The results for pigment production correspond to those obtained by Thimann et al (17) for Spirodela. However, they reported that glucose supported greatest growth and sucrose least, fructose again being intermediate in effect. As can be seen from the data in table I, the opposite growth effects are attributed to glucose and sucrose for these endosperm cultures. The same relative effectiveness of these sugars in the support of growth for endosperm cultures of a different variety of maize holds true (15).

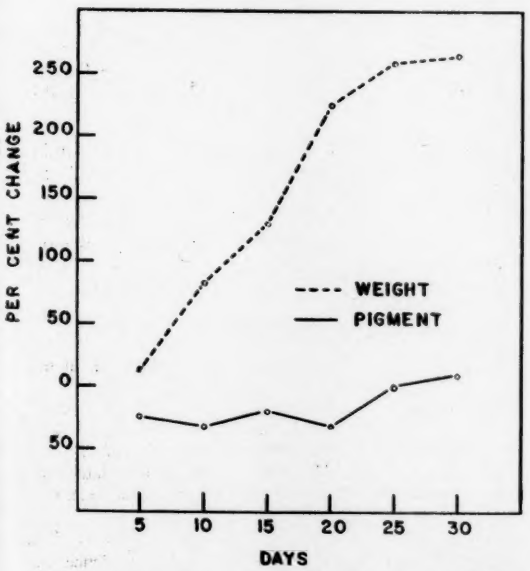
TABLE I

EFFECT OF SUCROSE, GLUCOSE, FRUCTOSE AND DIFFERENT CONCENTRATIONS OF SUCROSE ON ANTHOCYANIN FORMATION AND GROWTH *

SUGAR	CONC M	PIGMENT UNITS PER GRAM TISSUE	% INCREASE FRESH WEIGHT
Glucose	0.05	915	210
Fructose	0.05	1230	284
Sucrose	0.05	1520	268
Sucrose	0.10	1650	270
Sucrose	0.15	1840	260
Sucrose	0.20	1910	240
None	...	302	0

* Inocula for these experiments were taken from tissue grown in total darkness.

Increases in concentration of sucrose in the medium from 0.05 M to 0.2 M result in slight increases in pigment content. The increase in pigment does not appear to be commensurate with the increase in sugar concentration. While this appears to be true for Spirodela also, Thimann et al (17) have shown that there is a direct relationship between pigment content and reducing sugar content of Spirodela. However, the reducing sugar content of the tissue may not necessarily have a relationship to the sugar concentration in the medium.



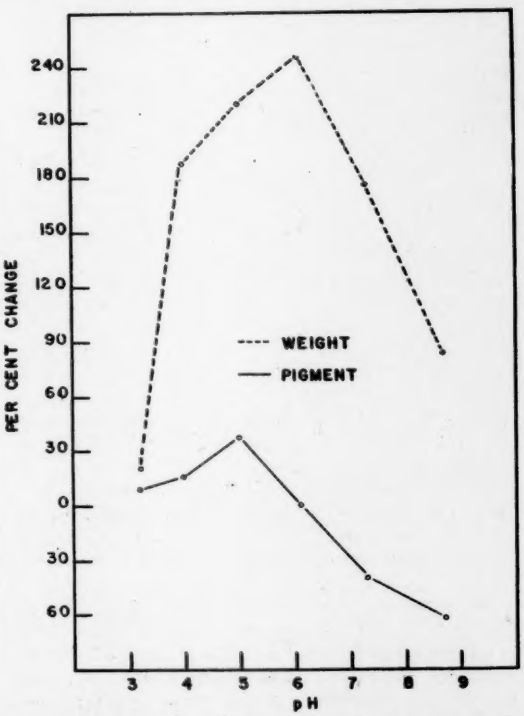


FIG. 1 (*left*). The growth-pigment curve. Pigment concentration changes very little during the growth period.
FIG. 2 (*right*). The effect of pH on pigment production and growth. Alkaline media cause a decided decrease in pigment formation.

Discussion

The effect of pH on pigment content is of considerable interest. With an initial pH of 3.2, growth of the tissue is negligible, yet the pigment content of the tissue has increased somewhat over what one might expect the initial pigment concentration to be (approximately 1500 pigment units per gram of tissue). At pH 8.7 some growth has occurred, yet the pigment concentration is only 570 pigment units per gram. Assuming an initial pigment concentration of 1500 pigment units and further assuming the tissue to have approximately doubled in bulk (87 % increase in wet weight), then the final pigment content of the tissue should be about 750 pigment units assuming no pigment synthesis has occurred. The value of 570 units would certainly indicate that no anthocyanin synthesis has occurred at pH 8.7. Indeed, there may have been some destruction of pigment. Approximately the same observations may be made of the tissue grown at pH 7.3. It would appear then that acid pH values down to at least 3.2 have relatively little effect on pigment production, while alkaline media definitely interfere with pigment synthesis or perhaps accelerate or induce the disappearance of the pigment. It is possible that highly alkaline media interfere with sugar uptake, but the fact that appreciable growth takes place indicates that sugar is entering the tissue. It is more probable that the high alkalinity has caused precipitation of some mineral element in the medium or otherwise restricted the entry of some mineral into the tissue which is needed for anthocyanin synthesis. In view of the work of Edmundson and Thimann (4) which implicated a copper-containing enzyme, this opinion does not seem unreasonable.

It is a general assumption that conditions which lead to the restriction of growth and which permit sugar accumulation are conducive to increased anthocyanin synthesis. The experiment with high pH would seem to indicate that caution should be used in applying such a generalization. Nevertheless, this assumption is borne out by other experiments reported here. If growth of the endosperm is inhibited by lack of sugar, pigment synthesis is very small. On the other hand, if growth is inhibited by low pH or by lack of organic nitrogen (from unpublished data), but sugar is supplied to the cultures, then pigment content reaches and frequently surpasses normal pigment concentration.

The effects obtained with sugars on pigment production are in general agreement with those obtained by most other workers. However, the results obtained with the sugarless medium require some comment.

The data reported in table I were obtained with inocula from tissue grown in the dark. Such tissue has a very low pigment concentration. When this tissue was transferred to a medium devoid of sugar, 302 pigment units per gram of tissue were synthesized in a 25-day experimental period. There are 2 possibilities to be considered as contributing to the pigment synthesis: 1) the tissue may have synthesized the pigment at the expense of stored carbohydrate since these tissues ordinarily store large amounts of starch on normal media, and 2) the concentration of anthocyanin represents pigment synthesized from a precursor formed in the dark but which requires light for the final step(s) in producing anthocyanin. Thus with the possible exhaustion of a precursor and with no carbohydrate available for its further synthesis, pigment production ceased. At any rate, one point which is clearly demonstrated is that light is essential for the synthesis of anthocyanin in these tissues and that the effect of light is not connected with the production of carbohydrates.

Summary

There are at least 3 anthocyanins produced by endosperm tissue cultures derived from 'Black Mexican Sweet Corn'. Two of them have been identified. One is cyanidin-3-monoglucoside (chrysanthemin) and the other is pelargonidin-3-monglucoside (callistephin). The 3rd pigment is present only in trace amounts and was not identified. In general acid media have small promotive effects on pigment synthesis, but alkaline media have a definitely inhibitory effect. It is suggested that the latter is due to the unavailability of copper or other mineral nutrients in alkaline media. Sucrose on a molar basis permits greater pigment synthesis than either glucose or fructose. Increased concentrations of sucrose have rather small effects on increasing pigment production. Light is essential for the synthesis of anthocyanin by these tissues. When grown in the dark, the tissue is virtually colorless. Such tissue shows visible signs of pigmentation after 6 hours exposure to light. It is suggested as one possibility that precursors are produced in the dark which require a photochemical reaction in order to be transformed into anthocyanin.

I wish to thank Professor K. V. Thimann for his aid and encouragement during the tenure of my fellowship.

The technical assistance of Mr. Andrew E. Anderson and Mr. Jerry R. Schwarz is gratefully acknowledged.

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AMIDE METABOLISM IN HIGHER PLANTS. III. DISTRIBUTION OF GLUTAMYL TRANSFERASE AND GLUTAMINE SYNTHETASE ACTIVITY[1]

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Previous reports (2, 16, 17) described the preparation and properties of a glutamyl transferase from pumpkin seedlings which catalyzes exchange of the amide group of glutamine with either hydroxylamine or isotopic ammonia. The product of the reaction is glutamyl hydroxamic acid or N^{15}-labelled glutamine, respectively. Cofactors for the exchange are manganous ion, adenosine polyphosphate, and either phosphate or arsenate. Similar enzyme preparations have been reported from bacteria, Neurospora, pigeon and mammalian tissues (7, 19, 20), and chick embryos (14). A glutamine synthetase which synthesizes glutamine from glutamic acid and ammonia, or glutamyl hydroxamic acid from glutamic acid and hydroxylamine, with adenosine triphosphate (ATP) as energy donor, has also been reported from various sources, including higher plants (5). Glutamine synthetase preparations from pea seeds have been shown to have transferase activity even after purification of 1000- to 4000-fold (6, 18). Levintow and coworkers (9) subjected a highly purified synthetase preparation from pea seed to ultracentrifugal analysis and showed that both synthetase and transferase activities were associated with a single monodisperse protein component. In preparations from several other sources, including pumpkin seedlings, they found a close association between synthetase and transferase. From this evidence it appears that the synthesis of glutamine and the exchange of the amide group are catalyzed either by a single enzyme or by 2 very closely associated enzymes. In view of the central role of glutamine in plant metabolism, it seemed desirable to learn something of the distribution of these enzymic activities in plants. If glutamyl transferase is of general importance in plant metabolism, or if it is in fact identical with glutamine synthetase, it should be widely distributed among plant species. If, on the other hand, it is found only in certain species, then the distribution might furnish the key to a role in

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some specialized phase of nitrogen metabolism. Cell fractionation appeared to offer a possibility of separating transferase and synthetase activities, since Webster (21) had reported glutamine synthetase to be associated primarily with the mitochondrial fraction of bean seedling homogenates.

This paper presents assays of glutamyl transferase activity in a number of species of plants and microorganisms, as well as data indicating that both transferase and synthetase activities are localized in the soluble cytoplasm, and that both behave similarly in isoelectric fractionation procedures.

Methods

Glutamine synthetase and glutamyl transferase activities were measured by the enzymatic formation of glutamyl hydroxamic acid in the appropriate reaction mixture. Composition of the reaction mixtures varied somewhat from one experiment to another, and details are given with each table. Essentially, the transferase assay measured formation of hydroxamic acid from glutamine and hydroxylamine by an exchange reaction, while the synthetase assay measured the formation of hydroxamic acid from glutamic acid and hydroxylamine with ATP as a source of energy.

Glutamyl hydroxamate was assayed colorimetrically by means of the red chelated complex which it forms with ferric ion (10, 15). One ml of 10 % trichloroacetic acid and 0.5 ml of a solution of 5 % $FeCl_3 \cdot 6H_2O$ in 95 % alcohol made 0.1 *N* with respect to hydrochloric acid were added to the reaction mixture, which was then diluted to 10 ml and filtered. The absorbence of the filtrate was measured with a Klett-Summerson or Bausch and Lomb Spectronic 20 colorimeter, at 540 mμ. Enzyme preparations from some plants were found to contain tannin-like substances which gave brown to greenish colors with ferric chloride. This interference could usually be eliminated by adding the trichloroacetic acid to the reaction mixture, diluting to 9.5 ml and filtering before adding the ferric chloride.

Glutamyl hydroxamic acid synthesized by the method of Roper and McIlwain (13) was used as a standard. The synthetic material had a melting point of 152 to 153° C, compared to a published value of 155° C. It gave colorimeter readings which were consistent with those obtained with succinyl hydroxamate used previously as a standard (8, 10, 17). The colorimetric assay follows Beer's law up to at least 10 micromoles (μM), and the components of the transferase system, in the quantities used, were shown not to interfere.

The investigation of glutamyl transferase distribution was carried out largely with acetone-dried materials as the source of the enzyme. In preparing these "acetone powders," plant materials were first washed in distilled water and then homogenized for about 2 minutes in a Waring blendor with 5 to 10 volumes of acetone chilled initially to −10° C. The suspension was filtered by suction in a Büchner funnel. The residue was then washed in the funnel, twice with cold acetone, once with acetone at room temperature, and 2 to 3 times with ether (USP) at room temperature. The resulting powder was dried for a few minutes in the air to remove ether, and then left overnight in a vacuum desiccator over phosphorus pentoxide to remove remaining traces of moisture and solvents. Cell-free enzyme preparations and suspensions of microorganisms were poured, with stirring, into 5 to 10 volumes of cold acetone and handled in the same manner. The dry powders were stored at −10° C. in tightly capped jars. Acetone powders prepared in this way have been found highly active after 20 months of storage.

Acetone drying seems to have no harmful effects, except to reduce the solubility of the enzyme. On the other hand, it provides material which can be stored conveniently and is free of plant pigments, which otherwise interfere in the colorimetric analyses. In the case of the microorganisms tested, no transferase activity could be detected in fresh cell suspensions, while acetone powders were highly active. This difference in activity was presumably due to destruction of the plasma membrane by solvents. Etiolated pumpkin seedlings were used to test the stability of transferase activity to the drying procedure. Ninety to 100 % of the activity was retained in the acetone powder, but only 50 % of it could be extracted with water, whereas the original enzyme obtained from fresh plant material was completely water soluble. Extraction with 1 % sodium bicarbonate brought 67 % of the activity into solution, but no method could be found for completely extracting transferase activity from the powder. For this reason, assays were performed by adding 5 mg of powder directly to each assay mixture. This amount of acetone powder was shown to have the same activity in assays as the equivalent amount of fresh extract.

For measurements of enzyme activity, a series of identical reaction mixtures, usually 3, was set up and allowed to react for different periods of time. A control, identical to the others but with glutamine omitted, was also included. The corrected readings were plotted against time, and the initial rate calculated in micromoles of glutamyl hydroxamate formed per hour.

Protein nitrogen was determined and used as a basis for comparison of activities. An appropriate sample of the acetone powder (usually 20 mg) was washed with 2 % trichloroacetic acid to remove nonprotein nitrogenous substances, and the residue was analyzed by the micro-Kjeldahl method. In some cases extracts or homogenates of fresh plant tissue were assayed. The same procedure was used, except that protein assays were generally made by a turbidometric method. An aliquot of the sample, containing 1 to 6 mg of protein, was diluted to 4.0 ml with water; 6.0 ml of 5 % trichloroacetic acid were added, and the turbidity was measured immediately in an Evelyn colorimeter at 540 mμ, against crystalline egg albumin as a standard.

In the species distribution studies sodium cyanide

was usually added to inhibit enzymatic browning. A wetting agent, Antarox, was sometimes added. Neither of these substances had any effect on transferase activity in pumpkin extracts. However, Antarox was found in some cases to increase the activity of acetone powders.

Cell fractions were obtained by differential centrifugation (1, 11). Plant materials were homogenized in the cold, with a buffered sucrose solution added in the proportion of 4 ml for each 3 g of tissue. The solution contained 0.1 M potassium phosphate buffer at pH 7.4 to counteract the rather high acidity of the tissues. Mung bean seedlings were homogenized with a solution containing phosphate buffer, 0.375 M sucrose, and 0.001 M ethylenediamine-tetraacetate. A solution containing phosphate buffer, 0.40 M sucrose, and 0.01 M sodium thioglycolate was used for homogenization of pea seedlings (var. Blue Bantam). Fractions were obtained by centrifugation of the homogenates at 4° C, and these fractions were then assayed for glutamyl transferase and glutamine synthetase activity.

Results

Glutamyl transferase activities of a number of species, calculated as described above, are presented in table I. In addition, transferase activity was demonstrated qualitatively in the following: the Thal-

Table I

Glutamyl Transferase Activity in Various Species *

Scientific name	Common name	Remarks	Activity**
THALLOPHYTA			
Rhodospirillum rubrum	purple bacterium	2-day culture	2
" "	" "	7-day culture	5
Clostridium kluyveri	bacterium		0
Chlorobacterium sp.	"		0
Saccharomyces cereviseae	bakers yeast		2
Chlorella sp.	green alga	grown on NH_4^+	9
Chlorella sp.	" "	grown on NO_3^-	4
Scenedesmus sp.	green alga		3
Irideae laminarioides	red alga		0
Ramalina reticulata	Spanish moss		0
SPERMATOPHYTA			
Gymnospermae			
Order Ginkgoales			
Ginkgo biloba	ginkgo tree	leaves	3
Angiospermae			
Order Ranales			
Magnolia soulangeana	magnolia	leaves	2
Liriodendron tulipifera	tulip tree	leaves	8
Order Caryophyllales			
Beta vulgaris	sugar beet	seedlings	2
Fagopyrum esculentum	buckwheat	seedlings	10
Order Polymoniales			
Lycopersicon esculentum	tomato	seedlings	8
Order Rosales			
Vicia atropurpurea	vetch	leaves	12
" "	"	cotyledons	8
Phaseolus vulgaris	garden bean	leaves	18
Medicago hispida	bur clover	leaves†	0-2
" "	" "	nodules†	40
Lupinus succulentis	lupine	nodules†	17
Lathyrus odoratus	sweet pea	nodules†	ca 60
Platanus acerifolia	plane tree	young leaves	0
Order Loasales			
Cucurbita pepo	pumpkin	seedlings	17
Echinocystis fabacea	manroot	leaves	52
Order Asterales			
Cynara scolymus	artichoke	leaves	2
Order Liliales			
Allium cepa	onion	bulb	13
Order Graminales			
Zea mays	sweet corn	seedlings	7

* The reaction mixture for rate measurements contained: Enzyme, 5 mg of acetone powder; buffer, 100 μM of sodium maleate, pH 6.4; glutamine, 20 μM; hydroxylamine, 20 μM; manganous sulfate, 1 μM; sodium arsenate, 20 μM; ATP, 0.2 μM; Antarox, when added, 0.1 ml of a 1 % solution, adjusted to pH 7 with sodium bicarbonate; sodium cyanide, when added, 10 μM. Total volume, 2.1 ml. Allowed to react 20 minutes before adding hydroxylamine. Temperature 38° C.

** Micromoles of glutamyl hydroxamate formed per hour per mg of protein.

† Indicates fresh suspension, instead of acetone power.

TABLE II

TRANSFERASE DISTRIBUTION IN THE PUMPKIN SEEDLING *

ORGAN	HYDROXAMATE FORMED (μM)	
	TOTAL OBSERVED	PER MG PROTEIN
Root	0.8	0.7
Hypocotyl	0.7	1.0
Cotyledons	2.6	1.3

* Reaction mixtures were as described in table I except there were only 10 μM of glutamine, and the total volume was 2.0 ml. There was no Antarox or sodium cyanide. Hydroxylamine was added 20 minutes after the other components, and the complete system was allowed to react 15 minutes at 37° C.

lophyta *Azotobacter agilis* and *Rhizobium trifolii;* the Pteridophyta *Equisetum telmateia* and *Polystichum munitum;* and the Spermatophyta *Sequoia sempervirens, Mesembryanthemum edule, Spinacia oleracea, Solanum tuberosum, Nicotiana tabacum, Phaseolus aureus, Lupinus succulentis* (stem and root), *Arachis hypogaea, Pisum sativum, Trifolium repens* (leaves and nodules), *Helianthus annuus, Baccharis pilularis, Chrysanthemum leucanthemum, Hordeum vulgare,* and *Avena sativa.* All of the legume nodules tested proved to have high transferase activity. In assays of *Rhizobium trifolii* grown in pure culture, no difference in transferase activity could be shown between a strain which produces nitrogen-fixing nodules and one which produces non-fixing nodules (strains R205 and R202 respectively, obtained from Dr. P. W. Wilson).

Table II shows transferase activities of the various organs of the pumpkin seedling. It will be noted that differences in protein content of the tissues account for a large part of the variation in activity. Table III shows similar data for older pumpkin plants. The tissues assayed here were all taken at the same time from the same plants. The young leaves were the 6th and 7th leaves above the cotyledons and were 2.5 cm or less in breadth. The old leaves consisted of the 3rd leaf above the cotyledons of each plant and were 10 to 12 cm broad. The cotyledons were 2 to 3 cm broad and apparently were still healthy and functional. No protein values were obtained for these plants, and the activities given are for 5 mg of acetone powder. However, it does not seem likely that the relatively higher activity observed in the old leaves is due to their having a higher protein content than the young actively growing leaves.

TABLE III

TRANSFERASE DISTRIBUTION IN THE PUMPKIN PLANT *

ORGAN	HYDROXAMATE FORMED (μM)
Cotyledons	0.8
Old leaves	3.6
Young leaves	1.9

* Assay conditions were as described for table I, except no Antarox or sodium cyanide was used, and the volume was 2.0 ml. Hydroxylamine was added 20 minutes after the other components, and the complete system was allowed to react for 5 minutes.

Tables IV and V compare the intracellular localization of glutamine synthesis and glutamyl transferase activity in 4-day-old mung bean and 3-day-old pea seedlings. The cell components can be identified tentatively on the basis of the centrifugal force used to isolate them. Centrifugation at 500 × G removes primarily nuclei and cell wall debris. Centrifugation at 20,000 × G precipitates mitochondria and other particles of similar size, while 78,000 × G precipitates microsomes in addition to the larger particles. Fifteen minutes centrifugation at 20,000 × G was found to leave the supernatant liquid slightly cloudy in the fractionation of mung bean. Therefore a portion of the homogenate was subjected to 60 minutes at this centrifugal force to yield a perfectly clear supernatant liquid. The precipitate obtained after 15 minutes centrifugation at 20,000 × G was shown to have Krebs cycle activity and thus is known to have contained mitochondria. The particles oxidized citrate actively, but malate was oxidized only after a 4 to 5 hour lag period. The malate oxidation was not due to bacterial contamination as Terramycin was added to inhibit bacteria.

TABLE IV

SYNTHESIS AND TRANSFER IN MUNG BEAN CELL FRACTIONS *

FRACTION	HYDROXAMATE FORMED (μM)	
	SYNTHESIS	TRANSFER
Homogenate, cleared at 500 × G	3.7	8.7
Supernatant, 15 min at 20,000 × G	4.4	9.3
Precipitate, 15 min at 20,000 × G	0.1	0.9
Same precipitate, washed	0.0	0.2
Supernatant, 60 min at 20,000 × G	4.1	9.3
Precipitate, 60 min at 20,000 × G	0.0	0.6

* Assay mixtures for synthesis contained: Enzyme equivalent to 300 mg of fresh tissue; Tris (hydroxymethyl) aminomethane buffer, pH 7.4, 120 μM; potassium glutamate, 200 μM; ATP, 6 μM; magnesium sulfate, 60 μM; sodium thioglycolate, 20 μM; hydroxylamine hydrochloride (neutralized with KOH), 200 μM. An additional 6 μM of ATP were added after 20 minutes. Transfer assay mixtures: Same enzyme as for synthesis; potassium maleate buffer, pH 6.4, 100 μM; manganous sulfate, 1 μM; glutamine, 20 μM; ADP, 0.2 μM; sodium thioglycolate, 20 μM; hydroxylamine hydrochloride (neutralized with KOH), 20 μM; potassium arsenate, pH 6.4, 20 μM. Total reaction time was 40 min, temperature 38° C, and volume 2.0 ml for both assays.

Similar fractionations of cell components were carried out with cotyledons from pumpkin and peanut seedlings. In pumpkin the transferase activity was

recovered in the supernatant liquid after 20 minutes at 21,500 × G (microsomes plus hyaloplasm). In peanut, centrifugation for 1 hour at 100,000 × G, to precipitate microsomes, left the transferase activity in solution. Glutamine synthesis activity of pumpkin seedlings seems to be very easily inactivated. Even in fresh homogenates, it could not be demonstrated consistently. Centrifugal fractionation of a pumpkin homogenate yielded high transferase activity, localized in the soluble fraction, but no significant synthetase activity in any of the fractions.

Previous work (17) indicated that the transferase activity of pumpkin seedling extracts was precipitated between pH 6.40 and pH 5.45. A similar fractionation was carried out with an extract from pea seedlings (the supernatant solution after 75 minutes centrifugation at 20,000 × G described above) to compare transferase and synthetase. No separation could be effected; although only 50 to 60 % of the activity was recovered, both transferase and synthetase activities were found in the fraction precipitating between pH 5.45 and pH 4.80.

Discussion

Webster (21) has demonstrated glutamine synthesis with homogenates of 9 species of higher plants. The data presented here show that glutamyl transferase activity is also widespread. Of 43 species tested for activity, only 5 failed to give any positive test. The species tested were chosen to give a wide cross-section and represent 8 orders of angiosperms as well as selected gymnosperms, pteridophytes, and thallophytes. They include classical "asparagine plants" as well as "glutamine plants."

The activity figures presented in table I should be regarded as minimum values. A low or zero value for a species may reflect the physiological condition of the sample or inadequacy of the assay method for that species. There is ample evidence that the activity is influenced by such factors as mineral nutrition, age of the plant, and the particular organ tested. In addition, any preparation with low adenosine triphosphatase activity would have shown low transferase activity under the conditions employed. After a large portion of the species distribution study had been completed, it was shown that adenosine diphosphate (ADP), rather than ATP, is the active cofactor (11). Apparently ATP is converted first to ADP, which then activates the system. Direct addition of ADP eliminates the lag period observed with ATP. Although the conditions employed gave maximum or nearly maximum activity with acetone powders of pumpkin seedlings, preparations from other plants might hydrolyze ATP more slowly, in which case the true transferase activity would be higher than shown here.

The data clearly demonstrate that glutamyl transferase activity is widespread in the plant kingdom and is not limited to any particular group of plants. There seems to be little doubt that the enzyme responsible for the transfer reaction is of general importance in plant metabolism. The greater transferase activity of mature leaves as compared to young, growing leaves is of interest, since one would expect tissues which are engaged in a rapid net synthesis of protein to have the highest rate of amide metabolism.

It was found here that both transferase and synthetase activities of pea seedlings and mung bean seedlings are localized in the soluble fraction of cell homogenates, corresponding to the amorphous cytoplasm. This finding is consistent with the suggestion that both activities are functions of a single enzyme. It is not in agreement with Webster's report (21) that the glutamine synthetase of bean seedlings is localized in the mitochondria. The similar behavior of the 2 activities during isoelectric fractionation is also consistent with the 1-enzyme hypothesis. However, reported differences in metal specificity are difficult to reconcile with this hypothesis. Manganous ion is most effective in the transfer reaction (2, 6, 16, 17), while magnesuim (6) or cobalt (3, 4) ions are most effective in glutamine synthesis.

Table V

Synthesis and Transfer in Pea Cell Fractions *

Fraction	Hydroxamate formed (μM)	
	Synthesis	Transfer
Whole homogenate	1.2	2.2
Supernatant, 75 min at 78 000 × G	1.4	2.1
Precipitate, 75 min at 78,000 × G	0.1	0.1
Supernatant, 255 min at 78.000 × G	1.2	1 9
Precipitate, 255 min at 78,000 × G	0.2	0.1

* Reaction mixtures were as described in table IV, except that no additional ATP was used in the synthetase system. Synthetase: 33 min at 37° C; transferase: 30 min at 38° C.

Summary

1. Glutamyl transferase activity was detected in 38 out of 43 species of higher and lower plants tested. The amount of activity found varied widely between species and even within single species and individuals.

2. Fractionation of cell components by differential centrifugation revealed that both glutamine synthetase and glutamyl transferase activities are associated with the soluble cytoplasm.

3. Glutamyl transferase and glutamine synthetase activities of pea seedling are both precipitated between pH 5.45 and pH 4.80.

4. The data obtained are consistent with the hypothesis that glutamine synthesis and the amide transfer reaction are catalyzed by a single enzyme.

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ISOLATION OF TWO QUINONES WITH COENZYME Q ACTIVITY FROM ALFALFA [1, 2]

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Several forms of coenzyme Q have been isolated from animals and microorganisms (3, 4, 7, 8). These compounds all are derivatives of 2,3-dimethoxy-5-methyl benzoquinone with a long side chain composed of monounsaturated isoprenoid units substituted in the 6 position (14). The convention has been introduced of using a subscript after coenzyme Q to indicate the number of isoprenoid units in the side chain. Thus coenzyme Q_{10} from beef heart has 10 monounsaturated isoprenoid units. The other forms of coenzyme Q which have been isolated are: Coenzyme Q_9 from *Torula utilis*, coenzyme Q_8 from *Azotobacter vinelandii*, coenzyme Q_7 from Torula, and coenzyme Q_6 from *Saccharomyces cereviseae* (8). No lower homologues of coenzyme Q have yet been prepared from natural sources. The observations of Prof. R. A. Morton and co-workers on coenzyme Q and a related compound called ubiquinone (11) have been discussed (3, 5).

When various plant materials were examined it was found that they contained 2 lipid soluble quinones, one with an absorption maximum at 275 mμ (coenzyme Q) and the other with an absorption maximum at 255 mμ which has been called Q_{254} (3). Since the designation Q_{254} may be misleading because of the use of subscripts to designate the members of the coen-

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zyme Q group, it is proposed that Q_{254} should be called plastoquinone (PQ). This name will serve to emphasize the localization of this compound in chloroplasts and possibly other plastid structures (3) as well as the fact that it is analogous rather than homologous with the coenzyme Q group in structure and enzymic function. The method of isolation and some of the chemical and enzymatic properties of these compounds obtained from dehydrated alfalfa will be described in this paper.

Methods

Purification of both coenzyme Q and plastoquinone was followed by spectrophotometric assay of the compounds in the extracts. Both coenzyme Q and plastoquinone are reduced by potassium borohydride to the respective hydroquinones. This reduction can be followed spectrophotometrically by observing the change in absorbence in the ultraviolet region which occurs when the peak corresponding to the quinone at 275 and 255 mμ respectively disappears with the concomitant formation of a peak with lower absorbence at about 290 mμ which is characteristic of the hydroquinone.

Since the isosbestic point for plastoquinone during reduction is at 275 mμ, all change in absorbence at 275 mμ is attributable only to coenzyme Q and thus coenzyme Q can be estimated directly from the change in absorbence at 275 mμ even in the presence of large amounts of plastoquinone.

The amount of coenzyme Q_{10} in a fraction can be calculated from the $\Delta E^{1\%}_{1cm}$ (oxidized minus reduced) at 275 mμ which has a value of 142, as determined from the measurements on purified material.

Since coenzyme Q shows an appreciable change in absorbence at 254 mμ during reduction, a correction for the amount of coenzyme Q present in a fraction must be made in the estimation of plastoquinone. The $\Delta E^{1\%}_{1cm}$ (oxidized minus reduced) for coenzyme Q at 254 mμ = 65, whereas the $E^{1\%}_{1cm}$ (oxidized minus reduced) at 254 mμ for plastoquinone = 198. The density change at 254 mμ minus 0.46 × density change at 275 mμ is the correct value for calculation of plastoquinone content.

To carry out the assay the fraction is dissolved in ethanol and the absorbence at 254 and 275 mμ is measured. Then a few grains of potassium borohydride are added; the solution is shaken and allowed to stand for 1 minute before determining the absorbence at 254 and 275 mμ again. From the changes observed the amounts of each compound can be calculated from the relationships cited above.

There appear to be 2 major compounds in alfalfa which interfere with spectrophotometric estimation of the quinones. The 1st is a carotenoid-like substance which shows a general increase in ultraviolet absorbence when borohydride is added to its alcoholic solution. The estimates of coenzyme Q and plastoquinone are low in presence of this impurity which is removed during the chromatography of extracts on decalso. The other major source of interference is more difficult to eliminate completely. Vitamin K_1 which is present in crude alfalfa extracts shows a decrease in absorbence at 275 mμ during reduction and a concomitant increase at 240 mμ. The isosbestic point is at 254 mμ. The reduction of vitamin K_1 proceeds more slowly than that of either of the coenzyme Q-active compounds so that the 1st rapid changes observed within 1 minute can be used as a rough estimate of coenzyme Q and plastoquinone. It has been found, however, that whenever large amounts of K_1 are present as indicated by increased absorbence at 240 mμ the estimates of coenzyme Q are usually somewhat high and the estimates of plastoquinone are somewhat low. Most of the K_1 is removed in the 1st stages of purification since this compound is usually eluted off decalso columns just before coenzyme Q and plastoquinone. It is difficult, however, to remove all traces of K_1, and it has been found desirable to start with extracts which contain only small amounts of vitamin K not only to improve the accuracy of estimation, but to facilitate the purification of the coenzyme Q and especially the plastoquinone.

The molecular weight of the compounds isolated has been determined by titration of the hydroquinones with the ferric chloride-*a,a*-dipyridyl reagent (12). The hydroquinones are prepared by reducing the quinone in ethanol (0.12 mg in 4 ml of ethanol) with a few grains of potassium borohydride. After reduction is complete the alcoholic solution is acidified by addition of 0.2 ml of 1.0 *N* hydrochloric acid and 0.4 ml of water and then extracted with 4.0 ml of *iso*-octane (2,2,4-trimethylpentane). The *iso*-octane phase is separated and the *iso*-octane evaporated. An appropriate amount of ethanol is used to redissolve the hydroquinone. The amount of hydroquinone in the ethanolic solution is determined by measuring absorbence at 290 mμ before and after reduction with borohydride. Any change at 275 or 255 mμ following addition of borohydride to the hydroquinone solution indicates the amount of quinone present. In a typical experiment 70 % of the added plastoquinone was recovered as hydroquinone and 8 % was recovered as reoxidized quinone in the final ethanolic solution. An aliquot of this ethanolic solution of the hydroquinone is then added to 3 ml of ferric chloride-*a,a*-dipyridyl reagent and the amount of iron reduced determined by measuring the increase in absorbence at 518 mμ as compared to an ethanol blank. An 0.1 μ equivalent of iron per ml gives an absorbence of 0.870 at 518 mμ in a cell with 1 cm path length.

Coenzyme Q activity was determined by the ability of the compounds to restore succinic oxidase activity in the electron transport particle (ETP) from beef heart mitochondria after coenzyme Q had been removed from the particle by an *iso*-octane extraction procedure which has been described (5). Briefly the extraction is carried out by shaking a suspension of ETP in 0.25 M sucrose containing 0.005 M versene and 0.2 M potassium acetate buffer (pH 5.5) with 4 volumes of 2,2,4-trimethylpentane (*iso*-octane) in

glass stoppered tubes on a reciprocal shaker for 1 hour at room temperature (25° C). After separation of the *iso*-octane phase the particles are washed by high speed centrifugation and placed under vacuum for 0.5 hour to remove residual solvent. Succinoxidase activity was determined manometrically as previously described (5). Only homologues of coenzyme Q and plastoquinone have been found to restore succinoxidase in extracted particles. Other lipid materials (13) which have been reported to stimulate cytochrome c reductase activity in particles subjected to less drastic extraction have no restorative effect on succinoxidase activity in particles from which coenzyme Q has been removed by the method described here.

Results

Both coenzyme Q and plastoquinone can be easily extracted directly from dried alfalfa with relatively non-polar solvents. They are also found in the non-saponifiable fraction obtained by extraction with long-chain hydrocarbons following saponification of alfalfa or spinach leaves in ethanolic KOH in the presence of pyrogallol. This saponification procedure is essentially the same as we have previously used for the purification of coenzyme Q from beef heart (4), and has been used successfully for the preparation of plastoquinone from both spinach leaves and dehydrated alfalfa.

For routine preparation of coenzyme Q_{10} and plastoquinone from dried alfalfa, direct extraction with long-chain hydrocarbons such as heptane or *iso*-octane is best. This procedure yields an initial extract containing most of the coenzyme Q material with only a slight amount of vitamin K_1. The direct extraction is also better adapted for large scale work than saponification followed by solvent extraction.

Chromatography on decalso and silicic acid has been found most useful for purification of the quinones contained in extracts obtained either by direct extraction or by extraction following saponification. When the extract is placed on either of these adsorbents in a hydrocarbon solvent the quinones are retained on the column while carotenes and waxy materials pass through. The quinones can then be eluted by slightly polar solvent mixtures which leave most of the residual impurities adsorbed on the column.

Purification of Plastoquinone and Coenzyme Q_{10} by Direct Extraction: The procedure used for the purification of both of these quinones is illustrated by the following example. Increased amounts of the compounds may be obtained by using the same procedure on a larger scale. The extracts from several batches of alfalfa may be pooled and chromatographed on proportionally larger columns.

Extraction and batch decalso absorption: Ten pounds of dehydrated alfalfa (National Alfalfa Dehydrating and Milling Co., Lamar, Colorado) were mixed with 1.5 gallons of heptane. Extraction was allowed to proceed for 48 hours. The heptane extract was removed by decantation, and an equal volume of Decalso (sodium aluminosilicate 50/80 mesh) was mixed with the extract. A yellow heptane solution was decanted leaving the coenzyme Q and plastoquinone among the materials adsorbed on the decalso. Elution of the quinones from the decalso was carried by the following solvent combinations: 1) 1500 ml heptane, 2) 3000 ml of 10 % ethyl ether in heptane, 3) 1500 ml of 15 % ether in heptane, 4) 1500 ml of 15 % ether in heptane and 5) 1500 ml of 20 % ether in heptane. Eluate 3 contained plastoquinone and eluates 4 and 5 contained a mixture of plastoquinone and coenzyme Q_{10}. A total of 40 mg of plastoquinone and 20 mg of coenzyme Q_{10} was recovered in these eluates. Occasionally some vitamin K_1 is found in the 1st eluates containing plastoquinone.

Extraction of ethanol soluble material: Evaporation of the heptane under reduced pressure from the eluates containing the quinones leaves a greenish-orange oil. When this oil was taken up in absolute ethanol a yellow solution with a brown precipitate was formed. The precipitate was filtered off and discarded. The quinones remain dissolved in the alcoholic solution.

Chromatography on decalso: The ethanol was evaporated and the yellow oil which remained was taken up in a small volume of *iso*-octane and the solution placed on a decalso column (6 cm × 20 cm, previously washed with *iso*-octane). Elution from the column with 100 ml of solvent collected for each fraction was carried out with the following solvents: 1) and 2), *iso*-octane; 3) to 7), 2 % ether in *iso*-octane; 8), 3 % ether in *iso*-octane. Essentially all of the coenzyme Q and plastoquinone was recovered in fractions 3, 4 and 5.

Silicic acid chromatography: The solvent was evaporated from the fractions containing the quinones, and the oil was taken up in a small volume of *iso*-octane and the solution placed on a silicic acid column (silicic acid 100 mesh: super cell 1 : 2 W/W, mixed and equilibrated with *iso*-octane 3 cm × 20 cm). The column was eluted under 4 lb psi nitrogen gas in 25 ml fractions with the following solvent mixtures: fractions 1 to 9, *iso*-octane; fractions 10 to 50, 30 % chloroform in *iso*-octane; fractions 51 to 61, 45 % chloroform in *iso*-octane. Fractions 12 and 13 contained 25 mg of plastoquinone and fractions 45 to 49 10.6 mg of coenzyme Q_{10}.

Second decalso column for plastoquinone: The best fractions of plastoquinone from the silicic acid column (fractions 12 and 13 above) were transferred to *iso*-octane as before and placed on a decalso column (3 cm × 15 cm washed with *iso*-octane). Eluate was collected in 100 ml fractions with the following solvent mixtures: Fractions 1 to 2, *iso*-octane; fractions 3 to 9, 2 % ether in *iso*-octane. Fractions 6 and 7 contained 20 mg of plastoquinone.

Crystallization of plastoquinone: The solvent was evaporated from the fractions containing plastoquinone and the residue was dissolved in 2 ml of hot absolute ethanol. A few white crystals may form as this solution cools. The solution was filtered and the

crystals discarded. The ethanolic solution was then set at −15° C overnight. Pale yellow crystals which form were collected by filtration. These crystals were recrystallized from ethanol at 5° C. Yield 10 mg, M.P. 42 to 43° C.

Second decalso column for coenzyme Q_{10}: The best fractions containing coenzyme Q_{10} from the silicic acid column (fractions 45 to 49) were taken up in *iso*-octane and placed on a decalso column similar to the one used for plastoquinone. Two hundred milliliter fractions of eluate were collected with the following solvent mixtures; fraction 1, *iso*-octane; fraction 2, 2 % ether in *iso*-octane; fraction 3, 5 % ether in *iso*-octane.

The 2 % ether eluate containing 6 mg of coenzyme Q was evaporated and the residue dissolved in 2 ml of hot ethanol. The solution was allowed to cool to room temperature and stand for 2 to 3 hours. If an orange oil forms at this point it should be removed by decantation of the mother liquor. The mother liquor is then set at −15° C overnight. It often occurs that more orange oil forms at −15° C along with orange crystals of coenzyme Q in the mother liquor above it. The mother liquor should be separated from the oil before filtering to obtain the crystals. Coenzyme Q was then recrystallized at −15° C overnight. If an excess of this orange oil is present it is desirable to rechromatograph the coenzyme Q fraction on decalso as decribed above before attempting the 2nd crystallization. Yield from the 2nd crystallization, 3 mg; M.P. 44 to 45° C.

Crystals from several batches were combined for recrystallization at 5° C in ethanol. The best crystals obtained had a M.P. 46.5 to 47.5° C and properties described below.

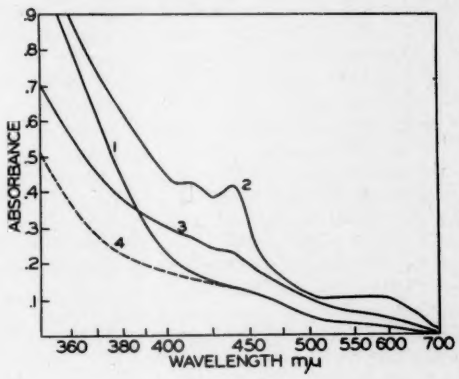
Discussion

Coenzyme Q isolated from alfalfa is identical in all respects (except for the 1 degree difference in M.P.) with coenzyme Q_{10} from beef heart (9). The spectrum shows peaks at 275 and 405 mμ and a minimum at 236 mμ. $E^{1\%}_{1cm}$ 275 = 162. $E^{1\%}_{1cm}$ 405 = 6.7. $\Delta E^{1\%}_{1cm}$ (oxidized-reduced) at 275 mμ = 137 and the ratio ΔE (oxidized minus reduced) to E oxidized at 275 mμ = 0.85. Following reduction with borohydride transient peaks appear at 248 and 312 mμ in the ultraviolet which may be taken either as evidence of semi-quinone formation or a color reaction based in part on the shift of the double bond in the side chain under alkaline conditions to a position conjugated to the ring, as has been suggested as the basis of the Dam-Karrar test.

When chromatographed on silicone treated paper this compound migrates at the same R_f as coenzyme Q_{10} from beef heart (10). A mixture of the 2 compounds shows no separation.

The molecular weight of the alfalfa coenzyme Q_{10} as determined by titration of the hydroquinone with ferric chloride and *a,a*-dipyridyl is 865, which agrees closely with the best estimates on coenzyme Q_{10} from beef heart (9).

Plastoquinone has a peak at 255 mμ (ethanol) in the ultraviolet ($E^{1\%}_{1cm}$ = 210). There is also a shoulder at 263 mμ and another shoulder in the visible around 425 mμ (cf fig 1). Concentrated solutions and the crystals show a pale yellow color. Upon reduction in ethanol with borohydride the peak at 255 mμ disappears and a new peak corresponding to the hydroquinone appears at 289 mμ−$E^{1\%}_{1cm}$ = 46. A slight shoulder remains in the visible above 400 mμ, whereas absorbence decreases from 425 to 308 mμ. During the early stages of reduction (immediately following KBH_4 addition) 2 peaks appear in the visible at 438 and 412 mμ. After standing for a few minutes these visible peaks disappear and the solution becomes distinctly greenish with the appearance of a band at 580 mμ. The 580 band slowly disappears and absorption in the visible returns to the original state after several minutes except for the decreased absorp-



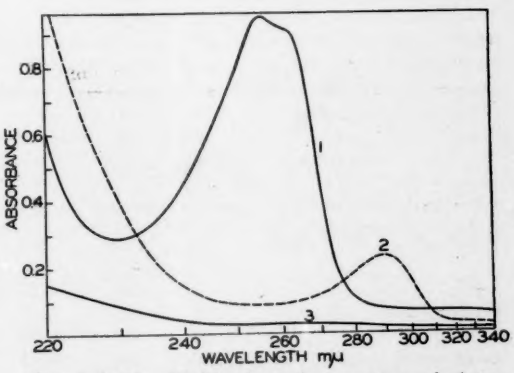


FIG. 1 (*top*). Visible absorption spectrum of plastoquinone. 1) 1.2 mg plastoquinone in 1 ml ethanol. 2) Same immediately after addition of a few crystals of borohydride. 3) Same 2 minutes after addition of borohydride. 4) Same after addition of more borohydride crystals. (*bottom*) Ultraviolet absorption spectrum of plastoquinone. 1) 0.043 mg plastoquinone in 1 ml. ethanol (path length 1 cm). 2) After reduction with a few grains of borohydride. 3) Cell blank with ethanol.

tion below 425 mμ which indicates the fully reduced compound as determined by the ultraviolet spectrum.

The properties of this compound are similar to those of a compound isolated from alfalfa by Kofler in 1946 (6). Kofler proposed on the basis of analysis of the spectral properties and a negative Cravens test a tetra-substituted benzoquinone derivative with unsaturated side chain(s).

Analysis of our compound gives C, 85.17 %, H, 10.75 % which is consistent with $C_{54}H_{82}O_2$ based on a molecular weight of 765 (theory C, 84.75 %. H, 11.07 %). The equivalent weight determined by titration of the hydroquinone with ferric chloride-dipyridyl reagent is 385, which corresponds to a molecular weight of 770 assuming 2 equivalents of iron per mole of hydroquinone. Since this compound has coenzyme Q activity, but no methoxyl groups (Zeisel), and a spectrum similar to duroquinone with a slight shift to lower wave length (analogous to the difference in absorption maximum shown in *iso*-octane by coenzyme Q_{10} (272 mμ) and eicosahydro-coenzyme Q_{10} (278 mμ)) (14) it may have a structure similar to 2,3,5-trimethyl benzoquinone with a side chain at the 6 position containing 9 monounsaturated isoprenoid units as in coenzyme Q_9. This hypothesis will be further tested by degradation and synthesis.

Both of these quinones show coenzyme Q activity in the restoration of succinic oxidase activity in *iso*-octane extracted beef heart mitochondria (5). The Q_{10} from alfalfa shows the same specific activity as beef heart Q_{10} and completely restores succinoxidase activity. Plastoquinone shows lower specific activity than Q_{10} and does not restore full activity. At higher levels plastoquinone appears to inhibit succinoxidase (cf. table I.) It is thus apparent that plastoquinone cannot fully replace coenzyme Q in its function and would appear to be a "pseudo"-coenzyme Q. Plastoquinone is unique, however, in that it is the only compound tested (5) which can substitute for the members of the coenzyme Q group in the restoration of succinoxidase after *iso*-octane extraction. It should be further noted that in the restoration of antimycin-sensitive succinate cytochrome c reductase activity which Ambe and Crane have described (1, 2) plastoquinone cannot replace the members of the coenzyme Q group (Q_8-Q_{10}).

In a previous paper we have reported that coenzyme Q_{10} in plant material is concentrated in mitochondria, whereas plastoquinone (Q_{254}) is found predominantly in chloroplasts (3). It may be expected therefore that the function of plastoquinone would be in photosynthetic electron transport. Hence, the assay for the coenzyme Q activity of this compound in a mitochondrial assay system would not be a proper measure of the unique properties of this compound.

TABLE I

COENZYME Q ACTIVITY OF ALFALFA QUINONES *

COMPOUND ADDED	SUCCINOXIDASE SPECIFIC ACTIVITY	INCREASE IN SPECIFIC ACTIVITY PER MG QUINONE	MG ADDED
Coenzyme Q_{10} (beef heart)	0.10	2.0	0.050
"	0.29	2.3	0.125
"	0.54	2.2	0.250
Plastoquinone	0.15	1.2	0.125
"	0.08	0.3	0.250
Coenzyme Q_{10} (alfalfa)	0.09	1.9	0.048
"	0.30	2.1	0.145
"	0.52	2.2	0.240
None	0.00	...	0.000

* Coenzyme Q activity determined as described under Methods. Coenzyme Q and plastoquinone added in a total volume of 0.05 ml ethanol; 0.10 mg beef heart mitochondrial phospholipid added to each flask containing 1.0 mg of enzyme in a total volume of 1.5 ml.

SUMMARY

Isolation and properties of 2 lipid-soluble quinones from alfalfa are described. The 1st with an absorption maximum at 275 mμ is identical with coenzyme Q_{10} isolated from beef heart and has the same coenzymatic activity. The 2nd quinone has an absorption maximum at 255 mμ and has been previously referred to as Q_{254}. The name plastoquinone is proposed for this 2nd quinone, instead of Q_{254}, to avoid confusion with the coenzyme Q group and to emphasize the fact that it occurs primarily in the plastids of green plants. This latter compound restores succinoxidase activity in the *iso*-octane extracted electron transport particle, but it is less efficient than authentic coenzyme Q, and inhibits activity at higher levels.

I wish to thank Professor D. E. Green for his encouragement of this research, Dr. R. L. Lester for kindly carrying out the paper chromatography of these compounds, and Mrs. Wanda Fechner for excellent technical assistance.

Note added in proof: Recently evidence has been published that a quinone from alfalfa, similar to plastoquinone if not identical with it, is 2,3-dimethyl-5-[3′-methyl-2′-butenyl-oktakis-(3′-methyl-2′-butylene)]-benzoquinone. (Trenner, N.R., Arison. B.H., Erickson, R.E., Shunk, C.H., Wolf, D.E. and Folkers, K. Coenzyme Q. VIII. Structure studies on a plant quinone. Jour. Amer. Chem. Soc. 81: 2026–2027. 1959).

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KINETICS AND PROPERTIES OF CYTOCHROME C PHOTOOXIDASE OF SPINACH[1, 2]

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Digitonin extracts of spinach chloroplasts have been shown to contain a cytochrome c photooxidase which can be separated into two components, designated Factor 1 and Factor 2 (4, 5). Both components are heat labile. Factor 1 is insoluble and contains chlorophyll. Factor 2 is water-soluble and has been extensively purified (5). The present paper describes the effects of light, temperature, salts, and inhibitors on the cytochrome c photooxidase system and summarizes observations on the distribution of the components of the system in photosynthetic and non-photosynthetic tissue.

METHODS

The methods used for preparing and assaying the activity of the components of cytochrome c photooxidase were the same as previously described (5). The preparation of Factor 2 was purified to the stage of the first active acetone precipitate (5). Changes in assay procedure were sometimes required, especially for determining the effect of varying light intensity, and will be noted in the appropriate sections.

The marine algae were obtained through the Supply Department of the Marine Biological Laboratory at Woods Hole, Massachusetts. The other green algae were cultured at 22° C with 4 % CO_2 in air under white fluorescent tubes providing an intensity of 4000 lux. The culture medium was the same as previously described (1). All the algae studied grew satisfactorily on this one medium.

The oats and barley were grown on well-washed mica (Vermiculite) with Hoagland's solution under the same light condition as described above. Normal cultures were used when 1 week old (after germination) and the etiolated preparations when 4 days old.

RESULTS

EFFECT OF VARIATION OF LIGHT INTENSITY: Several different methods of illumination were employed to determine the effect of light intensity on the rate of photooxidation of ferrocytochrome c. In the standard assay procedure described previously (5), the reaction mixture was illuminated from the top surface of a standard-size cell in the cell holder of a Beckman spectrophotometer. With this illumination system the light intensity was varied by use of a booster transformer and voltage regulator, and the

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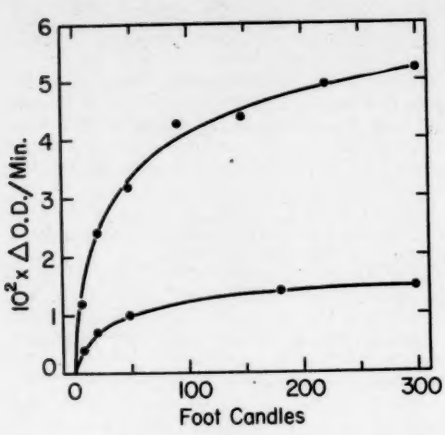

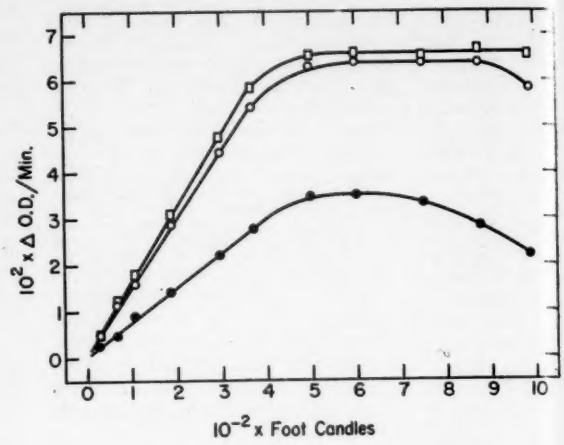

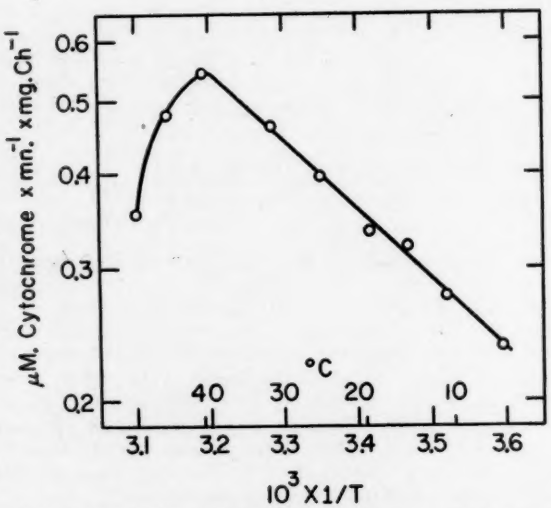

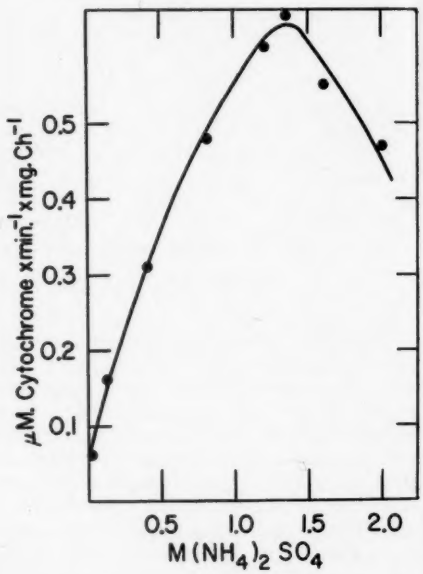

FIG. 1 (*upper left*). Variation of reaction rate with light intensity for the standard assay conditions. All reaction mixtures contained 1.3 M $(NH_4)_2SO_4$, 0.04 micromoles (μM) of reduced cytochrome c, and Factor 2 containing 0.16 mg protein, in a total reaction volume of 3 ml. Temperature, 20° C. Upper curve: Factor 1 containing 42 μg of chlorophyll; lower curve: Factor 1 containing 11 μg of chlorophyll.

FIG. 2 (*upper right*). Influence of light intensity on the cytochrome c photooxidase activity. Reaction mixtures contained 0.05 M phosphate buffer of pH 7.0, 1 M $(NH_4)_2SO_4$, 0.05 μM of reduced cytochrome c, Factor 2 containing 0.15 mg of protein, and Factor 1 containing chlorophyll as indicated in a volume of 3 ml. •——• = 30 μg chlorophyll; °——° = 60 μg chlorophyll; □——□ = 90 μg chlorophyll. Temperature, 20° C. Gas phase, air. White light.

FIG. 3 (*lower left*). An Arrhenius plot showing the temperature dependency of the rate of the cytochrome c photooxidase activity. The reaction system was identical to that described in figure 2, with 60 μg chlorophyll. Light intensity was 800 ft-c (white light). μM on ordinate represents micromoles.

FIG. 4 (*lower right*). Effect of $(NH_4)_2SO_4$ on the assay of cytochrome c photooxidase. The test system contained 0.1 M phosphate buffer of pH 7.0, 0.5 μM of KCN, 0.038 μM of reduced cytochrome c and Factor 2 containing 0.4 mg protein in a final volume of 3.0 ml. The amount of Factor 1 was adjusted to give convenient rates; temperature, 20° C; illumination, 40 ft-c. μM on ordinate represents micromoles.

intensity was measured at the surface of the liquid with a General Electric light meter (Model no. 8DW40Y16) registering foot-candles. Although it was clear that illumination through 3 cm of reaction mixture without stirring would give different reaction rates at different depths, the measurements of the variation of rate with light intensity had an empirical interest with relation to the standard assay system. Figure 1 shows some typical results. At 40 ft-c, the intensity employed in the photooxidase assay system (5), the reaction rate was approximately half of the maximum that could conveniently be achieved by this method. At light intensities above 300 ft-c, it was difficult to establish adequate shielding of the photocell.

In another set of measurements, a collimated beam of light from a 750-W projection bulb located 1 meter from the reaction cells was used for illumination after reflection by a first-surface mirror mounted at 45° above the reaction cell. A 15 cm cooled water cell was employed to filter the infrared from the light beam. Intensity was varied by placing calibrated neutral density filters in the light beam. A further modification of the method of illumination involved reflection of the light into a prism mounted in the 3rd and 4th positions of the cell holder. The partition between the 2nd and 3rd position of the holder was removed and the light coming through the prism entered the reaction cell through a polished third side. This method was advantageous when higher chlorophyll concentrations were used.

With the latter 2 methods, the rate of photooxidation of ferrocytochrome c increased linearly with light intensity up to about 400 ft-c, as shown in figure 2. Maximum rates were achieved at 500 ft-c. At higher light intensities there was a rate decrease which was more marked with lower chlorophyll concentration and probably reflected an inactivation by photooxidation of the enzyme system.

ACTION SPECTRUM: The effect of light of different wave lengths on the photooxidase was determined at 2 widely different intensities. Filters were inserted to isolate "red," "green," and "blue" light. The wave lengths of maximum transmission were 650 mμ, 530 mμ, and 420 mμ, respectively. For low light intensities the voltage regulator introduced in the light circuit was adjusted in each case so that the intensity of illumination measured with the General Electric light meter was 13 ft-c, which was well below saturation for unfiltered white light. Under the conditions of the standard assay, with the rate observed in 13 ft-c of white light taken as 100, the relative rate observed with 13 ft-c of red light was 133; with green light, 12; and with blue light, 29. At high light intensities a thermocouple with less wave length sensitivity than the photocell used, and a galvanometer, were employed to determine the relative intensities of essentially monochromatic light obtained through interference filters. Light intensity was varied by inserting calibrated neutral density filters until the galvanometer deflections were identical for each wave length investigated. The intensities used for 650 and 420 mμ were saturating. Under conditions of the standard assay procedure, the relative rates observed were 140 (650 mμ), 20 (530 mμ), and 130 (420 mμ). These figures show that the effective light was that absorbed by the chlorophyll. The relative ineffectiveness of the blue light at lower light intensities is probably due to the absorption of a relatively large proportion of this light by pigments inactive in the photooxidase reaction.

EFFECT OF TEMPERATURE: At 40 ft-c, with 28 μg of chlorophyll, the reaction rate increased linearly with temperature from 5° to 30° C. The apparent linear relationship was probably due to the fact that the light intensity was partly rate-limiting. This was borne out by observations made at 800 ft-c with 60 μg chlorophyll over a temperature range from 5° to 50° C. Figure 3 shows an Arrhenius plot of the data, illustrating the linear decrease of the log of the rate with the reciprocal of the absolute temperature. The experimental activation energy (ΔE_{exp}) was calculated as 4.3 kcal/mole. Thermal inactivation, accompanied perhaps by photo-inactivation, began about 40°.

EFFECTS OF SALTS AND pH ON REACTION RATE: The effect of $(NH_4)_2SO_4$ on the reaction rate of cytochrome c photooxidase is shown in figure 4. With increasing salt concentration the reaction rate rises sharply to reach a maximum at about 1.3 M. Above this concentration the rate decreases and becomes zero at 3 M $(NH_4)_2SO_4$ (not shown on the curve). The ratio between the rate at 1.3 M $(NH_4)_2SO_4$ and the rate in the absence of $(NH_4)_2SO_4$ was about 10 under the conditions used for the experiment of figure 4. In these measurements cyanide was used to inhibit the dark oxidation of ferrocytochrome c. This was necessary at low concentrations of $(NH_4)_2SO_4$ (below 0.1 M), but at higher concentrations the salt itself inhibited the dark oxidase so that the photooxidase activity could be measured in the absence of cyanide. Comparison of the rates observed in the presence of $(NH_4)_2SO_4$ and potassium cyanide with the rates observed in the presence of $(NH_4)_2SO_4$ alone showed that cyanide at a concentration of 0.3 mM stimulated the rate of the photooxidase by about 40 to 60 %, at all $(NH_4)_2SO_4$ concentrations tested (from 0.1 to 1.7 M). Increasing the cyanide concentration to 3 mM gave a stimulation only 0 to 10 % greater than that observed with 0.3 mM cyanide.

The reaction rate of cytochrome c photooxidase did not change with pH when this was varied (at intervals of about 0.5) from pH 5.2 to 8.7 by the use of NH_4OH—$(NH_4)_2SO_4$ mixtures, provided the concentration of sulfate was kept constant at 1 M. This suggested that the divalent anion, SO_4^{--} was primarily responsible for the stimulatory effects of the $(NH_4)_2SO_4$.

The effects of phosphate on cytochrome c photooxidase are shown in figure 5. All measurements at low (< 0.1 M) phosphate concentration were made

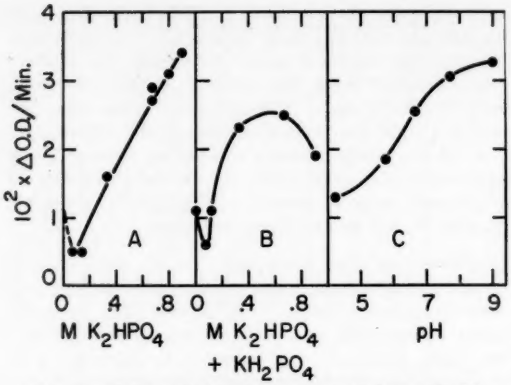

FIG. 5. Effect of phosphate concentration and pH on cytochrome c photooxidase activity. All measurements were made with the same enzyme preparations, Factor 1 containing 42 μg of chlorophyll, Factor 2 containing 0.5 mg of protein, 0.05 μM of reduced cytochrome c, plus phosphate as indicated, all in a volume of 3 ml. Illumination, 40 ft-c; temperature, 20° C. Rates at 0 phosphate were measured in the presence of 0.3 mM KCN. A. Effect of increasing molarity of K_2HPO_4. B. Effect of increasing phosphate at constant pH. Equimolar amounts of K_2HPO_4 and KH_2PO_4 were used to give a pH of about 6.7. C. Effect of varying pH at constant total phosphate concentration of 0.67 M.

in the presence of potassium cyanide at a concentration of 0.3 mM, to inhibit the dark oxidase. As in the case of $(NH_4)_2SO_4$, higher concentrations of phosphate inhibited the dark oxidase so that cyanide addition was not necessary for measurement of the photooxidation rate. Cyanide did not stimulate the reaction in the presence of phosphate, however, so that the curves showing the phosphate effects were identical whether cyanide was present or not at the higher phosphate concentrations. Another difference between sulfate and phosphate was the inhibition observed with small amounts of phosphate. Figure 5 A shows that the first effect of adding K_2HPO_4 to the photooxidase reaction mixture was an inhibition which reached a maximum at about 0.07 M phosphate. At this concentration the rate was only about one-third of that observed in the presence of 0.3 mM KCN and either 0.007 M K_2HPO_4 or 0.007 M $(NH_4)_2SO_4$. When the concentration of K_2HPO_4 was increased above 0.07 M, a strong stimulation was observed. Increasing total phosphate concentration at a constant pH of 6.7, maintained by using equimolar amounts of K_2HPO_4 and KH_2PO_4 gave the results shown in figure 5 B. The curve shows the same initial inhibition at low salt concentrations, but there is an activation at higher concentrations with a maximum effect at about 0.67 M phosphate. When the total molarity of phosphate was maintained at 0.67 M and the pH was varied by changing the ratios of the 2 salts, K_2HPO_4 and KH_2PO_4, the S-shaped curve shown in figure 5 C was obtained. This is reminiscent of a pH-titration curve. It seems likely that the pH effect observed reflects the fact that the divalent anion, HPO_4^{--} is a better stimulator than the monovalent anion, $H_2PO_4^-$.

The effects of adding NaCl, $NaNO_3$, and Na citrate were also studied. Some of the results are assembled in table I, which includes, for comparison, the rate observed with 0.3 mM KCN alone, the lowest rate (with cyanide and 0.07 M phosphate), and the highest rate (with cyanide and 1.5 M $(NH_4)_2SO_4$). All salts gave a stimulation, but citrate was more effective at concentrations below 1 M, whereas nitrate and chloride had relatively little effect below 3 M. Cyanide was not stimulatory when tested with chloride, nitrate, or citrate. Thus the stimulation by cyanide appears to be limited to the reaction carried out in the presence of sulfate. Attempts were made to test a number of salts of divalent cations, such as $MgCl_2$, $BaCl_2$, and $CaCl_2$. The formation of precipitates made accurate assays impossible, but no evidence was obtained for any stimulation by divalent cations.

The effect of urea on cytochrome photooxidase was different from that of salts. Urea did not inhibit the dark oxidase even at high concentrations and was, therefore, tested in the presence of cyanide. From 0.6 M to 3.0 M urea caused approximately 50 % inhibition of the photooxidase. Higher concentrations of urea elicited a rapid dark oxidation of ferrocytochrome c which was not inhibited by cyanide, and was not further examined.

TABLE I

EFFECT OF SALTS ON ACTIVITY OF CYTOCHROME C PHOTOOXIDASE

SALT	MOLARITY	0.3 mM KCN	RATE * $10^3 \times \Delta$ O.D. PER MINUTE
...	...	+	12
K_2HPO_4	0.07	+	5
$(NH_4)_2SO_4$	1.5	+	60
NaCl	1.3	−	10
"	2.7	−	11
"	3.6	−	18
"	3.6	+	18
$NaNO_3$	0.22	+	10
"	1.1	−	13
"	2.2	−	10
"	4.5	−	20
"	6.0	−	32
"	6.0	+	31
NaCitrate	0.033	+	12
"	0.17	−	18
"	0.33	−	25
"	0.67	−	31
"	0.77	−	31
"	0.87	−	31
"	0.87	+	31

* Corrected for the rate of dark oxidase, if necessary.

INHIBITORS: Known inhibitors of photosynthesis were tested on the cytochrome c photooxidase with 0.05 M phosphate buffer (pH = 7) and 1.0 M $(NH_4)_2SO_4$ added to the assay reaction mixture. Under these conditions the reaction rate was almost maximally stimulated so that any possible activating action of the compounds studied might not have been observed. Corrections for dark rate were always made.

Phenyl urethane, *o*-phenanthroline, dicumarol, 3(3-4 dichlorophenyl) 1-1 dimethyl urea and 3(3-chlorophenyl) 1-1 dimethyl urea were non-inhibitory when used at a concentration one-third that of a saturated solution. Hydroxylamine, sodium azide and potassium cyanide over the concentration range 3.3×10^{-4} to 3.3×10^{-2} M were also non-inhibitory. Other typical inhibitors such as sodium arsenate, sodium arsenite, dinitrophenol, carbon monoxide, iodoacetate and various chelating compounds were also ineffective as poisons. None of the following cations (as chloride salts) acted as inhibitors: Mn^{++}, Mg^{++}, Co^{++}, Fe^{++}, Fe^{+++}, Cu^{++}, Ni^{++}, Zn^{++}, and Ca^{++}. Tests with Ca^{++} and Mg^{++} were complicated by precipitates, but in short time studies no apparent inhibition was observed. The only heavy metals that inhibited the reaction were Hg^{++} and, to some extent, Al^{+++}. Fifty percent inhibition was produced by 4×10^{-5} M $HgCl_2$, 2.5×10^{-5} M *p*-chloromercuribenzoate and 3×10^{-4} M phenylmercuric nitrate. No attempt was made to reverse this type of inhibition by such well-known methods as glutathione addition, etc., since such compounds would reduce the cytochrome.

TABLE II

CYTOCHROME c PHOTOOXIDASE DISTRIBUTION IN HIGHER AND LOWER PLANTS

PLANT	PHOTOOXIDASE ACTIVITY	
	CRUDE EXTRACT	PURIFIED EXTRACT
Higher:		
Spinach	+	+++
Sugar beet	+	+++
Turnip leaves	+	+++
Radish "	+	+++
Mustard "	+	+++
Beet "	+	+++
Barley Green	+	+++
Barley Etiolated	−	−
Oats Green	+	+++
Oats Etiolated	−	−
Fern (*Pteridium latiusculum*)	−	−
Lower:		
Chlorella vulgaris	+	+++
Chlorella pyrenoidosa	+	+++
Scenedesmus obliquus	+	+++
Chlamydomonas moewusi	+	+++
Ankistrodesmus braunii	+	+++
Fucus sp.	+	+++
Laminaria sp.	+	+++
Ulva sp.	+	+
Enteromorpha sp.	+	+

DISTRIBUTION OF THE COMPONENTS OF CYTOCHROME c PHOTOOXIDASE IN NATURE: Factor 1 could be obtained from chloroplasts of practically any green plant material. Of course, this fraction would not be expected to occur in sources devoid of chlorophyll. In preparations of certain marine algae, the amount of Factor 1 obtained by the conventional method was relatively small. Higher concentrations of digitonin sometimes increased the yield. Preparations of Factor 2 from such material were consequently assayed with Factor 1 from spinach, since Factor 2 from different sources could be used interchangeably with other sources of Factor 1, and vice versa.

Several different types of photosynthetic organisms were examined for their content of Factor 2. For these tests, acetone precipitates were prepared by the procedure used for spinach. For the calloused tissue of the marine algae, some modifications on the method of preparation of extracts were necessary. Primarily these entailed the rupturing of the thalli in a modified Hughes bacterial press or the preparation of powders of the thalli by grinding in dry ice prior to the digitonin extraction. The results are summarized in table II.

A number of non-green tissues were tested for the presence of Factor 2. Extracts were prepared by the same procedure used for spinach. The acetone precipitates were extracted with relatively small volumes of buffer to give a large concentration of solids for a given volume, and tested with Factor 1 of spinach both before and after dialysis. Dialysis was often necessary to remove material which reduced cytochrome c in the dark. Dried baker's yeast, lamb liver and carrot root gave negative results for Factor 2. Green peas and wheat germ gave a trace of activity of questionable significance. White turnip roots gave positive results. On a fresh weight basis, the turnips contained about one-tenth as much activity as spinach. Factor 2 of turnip roots was heat labile, non-dialyzable and considerably less stable, on storage, than Factor 2 of spinach leaves. The stability was so different from that of the leaf preparations as to suggest that Factor 2 of turnip root might be a very different type of material from Factor 2 of leaves.

DISCUSSION

The dependence of cytochrome c photooxidase on a relatively labile chlorophyll-containing component, and on another component which is both heat labile and non-dialyzable supports the view that these components represent functional fragments of the photosynthetic machinery of the chloroplast. The observed distribution of Factor 2 also supports this view. The questions arise: "How and in what manner do these components interact, and what is the relationship of these reactions to the metabolic processes in the leaf?" The experimental data do not permit definitive answers to these questions. It appears permissible, however, to state our current working hypothesis. This can best be done with the aid of the diagram

shown in figure 6, which includes all of the various reactions which cytochrome c may undergo with chloroplasts, i.e., photoreduction, photooxidation, and dark oxidation (4). The numbers represent oxidation-reduction reactions, the nature of which may be deduced from the reactants connected by the lines. Thus 1a and 1b represent the oxidation-reduction reactions undergone by the photoactivated chlorophyll, either alone, or in conjunction with other compounds to give an oxidant (Ox.) and a reductant (Red.).

The reaction whereby oxygen is generated is represented by 2; 3 and 4 represent reaction(s) whereby ferrocytochrome c is oxidized by (Ox.), and 5 represents reaction(s) whereby ferricytochrome c is reduced by (Red.). Reaction 6 represents the cyanide-sensitive port of entry for the oxygen in the dark cytochrome oxidase reaction, which would then have reaction 4 in common with the photooxidase. The data do not *require* that these reactions have a common path, but the scheme is intended to show how this might occur. Reaction 7 represents the entrance point for oxygen in the photooxidase. When the chloroplasts are photoreducing cytochrome c, reactions 2 and 5 (which may be linked in a manner not indicated on the diagram) occur so rapidly that reactions 3, 4, and 7 are masked. Digitonin would interfere in some way with reactions 2 and 5. Factor 1 contains the chlorophyll and would function in reactions 1a, 1b, 3, 4, and 6, but not reactions 2 and 5. Factor 2 may act to facilitate reaction 7 or reaction 3.

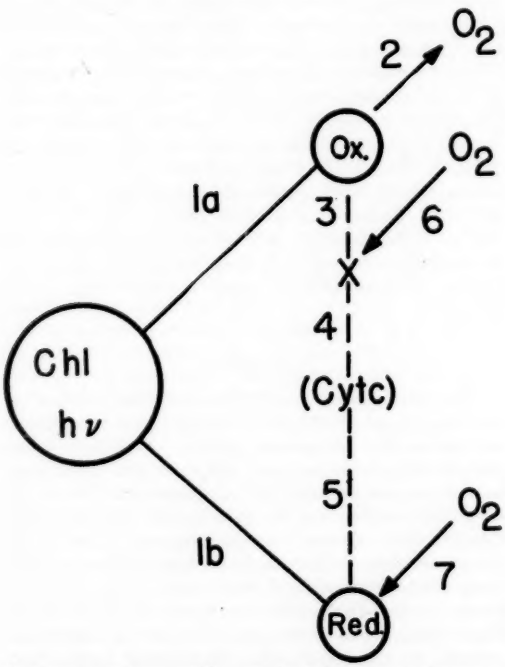

FIG. 6. The possible relationship of cytochrome c photooxidase to the photosynthetic apparatus.

The above representation is not the only way in which the interactions of the components of the system can be represented. In particular, it seems that the simultaneous loss of reactions 2 and 5 may imply a closer association between these reactions than is indicated in the diagram. The reductant for the Hill reaction may be a product of the oxygen evolving step, rather than the substance (Red.) which is represented as the reductant for oxygen in the diagram.

It is possible that the cytochrome c photooxidase represents a component of an oxygen-requiring respiratory process which functions during a recovery phase following oxygen evolution as pictured by Warburg (6). A strong argument against this view is the slow rate of the reaction. The maximum rate of cytochrome c photooxidation is not much greater than the rate at which a green cell respires in the dark, and the conditions of very high salt concentration required to elicit this rate appear to be highly abnormal. Also, the physiological cytochrome c concentration in or at the surface of the chloroplast is probably very low. Estimates of the rate at which this photooxidase might cause "photooxidations" with intact chloroplasts yield values in the range of 1 to 10 micromoles of oxygen per mg chlorophyll per hour. Such estimates take into consideration the fact that Factor 2 appears to be in short supply in the chloroplast, though more abundant in the cytoplasm. However, if we consider the not unreasonable possibility that cytochrome c from horse heart may not represent the true substrate of this enzyme system, then the low rates observed with the photooxidase are not necessarily disturbing. Additional evidence for a functional role for the photooxidase lies in the demonstration that spinach chloroplasts can cause a photophosphorylation dependent on the oxidation by oxygen of reduced dichlorophenol indophenol. The photophosphorylation rate is about as fast (40 micromoles P esterified per mg chlorophyll per hour) as the dark respiratory rate of the leaves (2). Under similar conditions, a photophosphorylation dependent on added cytochrome c and oxygen can also be demonstrated, with a rate of about 1 micromole phosphate per mg chlorophyll per hour[4]. Marré and Servettaz (3) and Wessels (7) have also reported a stimulation of photophosphorylation by cytochrome c.

SUMMARY

A study has been made of the properties of the cytochrome c photooxidase system of spinach. Light saturation curves have been determined. Measurement of the relative effectiveness of light of different wave lengths showed that the effective light was most probably absorbed by the chlorophyll of the pigmented component (Factor 1). From rate measurements at different temperatures, an experimental activation energy was calculated of 4.3 kcal per mole.

[4] T. Nakamoto and A. Marcus, unpublished observations.

The stimulation of the photooxidase by ammonium sulfate and potassium phosphate was examined in some detail. Maximum stimulation was as much as 10-fold in 1.3 to 1.5 M $(NH_4)_2SO_4$ in the presence of 0.3 mM KCN. Part of this stimulatory effect was due to the cyanide. Phosphate and citrate also stimulated strongly, and salts of monovalent anions had smaller effects.

Many typical inhibitors of photosynthesis have been shown to be without effect on cytochrome c photooxidase. The only effective inhibitors found were $HgCl_2$, *p*-chloromercuribenzoate, and phenylmercuric nitrate.

A survey of the distribution of the photooxidase in nature has shown that all chlorophyll-containing tissues examined contain both of the components (i.e., Factor 1 and Factor 2) of the photooxidase, and that these 2 factors interact to give an active photooxidase, regardless of the source from which they are prepared. The soluble component, Factor 2, has been shown to occur primarily in tissues containing chlorophyll.

The authors are grateful for the many stimulating discussions with Dr. Hans Gaffron.

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ACTIVATION OF GERMINATION IN DOUGLAS FIR SEED BY HYDROGEN PEROXIDE [1, 2, 3]

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The stimulating effect of hydrogen peroxide (H_2O_2) on seed germination and subsequent seedling growth has been noted in a number of species, e.g., eastern white pine (21), legumes (2), tomato (1), barley (12, 27), and others (17). Recently a rapid viability test based on this stimulating effect was developed for the determination of coniferous tree seed quality. Test results were found to be significantly correlated with germination results (7). Since H_2O_2 is active on seeds with different food reserves, e.g., starchy (barley), starchy and proteinaceous (legumes), and fatty (coniferous), a knowledge of the mechanism by which the chemical activates growth would help to elucidate the process of seed germination in general.

The very first sign of germination of a seed is the rapid increase of respiration which often starts within 1 hour after the commencement of water imbibition (24, 25). A study of the effect of H_2O_2 soaking on seed respiration during early stages of germination might shed some light on the mechanism of the activation.

Materials and Methods

Since ecotypic variations in germination and respiration have been observed in plants, 2 different seed lots of Douglas fir, (*Pseudotsuga menziesii* (Mirb.) Franco) (through the courtesy of Mr. Royce Cornelius, Weyerhaeuser Timber Company, Centralia, Washington) were used in the study. One lot (lot I) was collected in 1957 at the White River Tree Farm, Enumclaw, Washington at an elevation of 3,000 to 3,500 feet. After processing, this lot was stored in sealed metal cans at 3° C until used. The other lot (lot II) was collected in 1956 at the Vail Tree Farm

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[2] Technical paper 1200. Oregon Agricultural Experiment Station.

[3] The project was conducted cooperatively with the Northwest Forest and Range Experiment Station of the Forest Service, the United States Department of Agriculture, the Oregon Forest Protection and Conservation Committee, and the Forest Tree Seed Committee affiliated with the Western Forestry and Conservation Association.

near Elbe and Mineral, Washington at 1,500 to 2,000 feet elevation. After processing, lot II was preserved in sealed cans at −18° C for 1 year, then transferred to 3° C until used. The 2 lots of seed were further processed to separate out the light and the extremely heavy seeds during a South Dakota Blower (3). Lot I after blowing contained 8.80 % moisture on a fresh weight basis, the average weight per seed being approximately 11.5 mg. Lot II had 8.70 % moisture and weighed about 12.9 mg per seed. This paper will report the detailed experimental findings of lot I, since lot II responded similarly in the mode of action and only somewhat differed in magnitude.

The effect of H_2O_2 on germination was studied by soaking duplicate samples of 100 seeds each in 100 ml of water or 1 % H_2O_2 solution for 12, 24, 36, and 48 hours at 20° to 25° C. Following soaking, the seeds were planted in plastic boxes containing moist sand. The boxes were incubated in a germinator at 20° to 30° C (alternating 30° C for 8 hours with fluorescent light and 20° C for 16 hours in darkness). Seeds with radicle emergence of 1 mm or more were counted as germinated. Counts were made at intervals of 1 to 5 days. Germination of air-dried seeds and stratified (soaked in tap water for 24 hours and then chilled at 3° C for 10 days) seeds also were observed for comparison.

Gas exchange studies were performed in a circular Warburg respirometer at 30 ± 0.5° C. Carbon dioxide evolution was measured by Warburg's direct method (28) while oxygen uptake was measured on a duplicate sample in a flask containing KOH in the center well. The respiratory studies were conducted at 3 separate stages of germination because of the variability and slowness in the rate of development which is characteristic of tree seeds (23).

Respiration of Dry Seeds and at the Stage of Water Imbibition: Duplicate 100 air-dried seeds for each lot were weighed and put in Warburg flasks of approximately 15 ml in volume. Gas exchange of the dry seed was measured at the end of 24 hours. After the pressure changes had been recorded, 1.5 ml distilled water was added into the side arm of each flask and the vent closed after 15 minutes of shaking. The water was then tipped in 5 minutes later, and readings were taken at hourly intervals up to 5 hours. The bound CO_2 in the seed was not checked since Stanley found that it is negligible in the germinating sugar pine seed (23).

Effect of H_2O_2 on Respiration of Imbibed Seed: Duplicate 100 seeds for each lot were soaked in 100 ml of water or 1 % H_2O_2 for 6, 12, 24, 36, or 48 hours. Treated seeds were rinsed several times with water, surface-dried, and the fresh weight determined for each sample to permit calculation of the flask constants by using the specific gravity of the seeds. Seeds were put in Warburg flasks of approximately 15 ml in volume, and 0.5 ml of distilled water was added to each flask to maintain a constant relative humidity. After a 30-minute equilibration period, readings of pressure change were taken at hourly intervals for 4 hours.

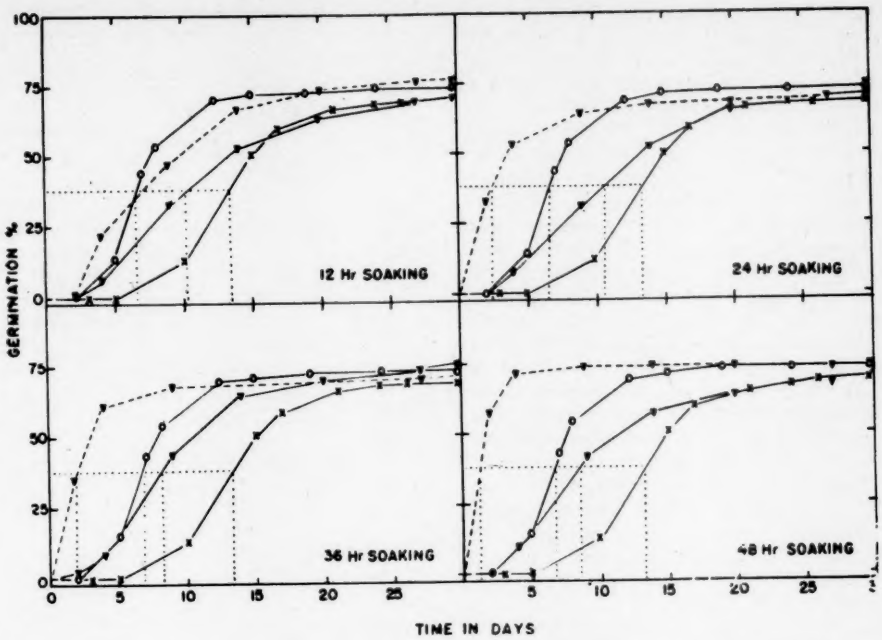
Respiration of Seedlings from Seed Soaked with H_2O or H_2O_2: Four replications of 100 seeds for each lot were soaked in 100 ml of H_2O or 1 % H_2O_2 for 36 hours, and each sample was then germinated at 20° to 30° C. On the 5th and 6th day of incubation, seedlings from seeds soaked with H_2O_2 were separated according to their size into 4 groups: A. Seedling with radicle 1 to 5 mm in length; B. Seedling with radicle 6 to 10 mm; C. Seedling with radicle 11 to 25 mm, and D. Seedling with radicle 30 to 40 mm and with epicotyl arch but no visible cotyledon expanding. Seedlings with expanded cotyledons were discarded as photosynthetic tissue was not included in the present study. Seedlings from H_2O soaked seeds were examined on the 9th and 10th day of incubation, as this period coincides with the 5th and 6th days incubation of H_2O_2 soaked seed insofar as the cumulative germination percentage was concerned. Duplicate 10 seedlings from each seedling group for each treatment were selected, rinsed, and surface-dried for the determination of respiratory rate as outlined above.

At the end of each respiration study, each sample was surface-dried, and its dry weight and moisture content was determined by drying to constant weight at 85° C in a forced-air oven. Each experiment on germination, respiration, and moisture determination was repeated 2 to 6 times.

Results

Mode of Activation in Germination: The average cumulative germination percentages of lot I with different pretreatments are summarized in figure 1. It is clearly indicated that germination is hastened by H_2O_2 soaking, and the increase is correlated with duration of soaking. Twelve hours soaking with H_2O changed the time required to obtain 50 % of the total germination potential from 13.5 days in air-dried seeds to 10.5 days, whereas H_2O_2 soaked and 10 days stratified seeds needed 7 days to produce the same percentage of germinated seedlings. In seeds soaked with H_2O for 24, 36, and 48 hours, the time required to obtain 50 % of the total germination potential was 10.5, 8.5, and 8.5 days respectively, whereas corresponding soaking with H_2O_2 altered the time to 2.5, 2.0, and 1.5 days respectively. This increase in germination speed was also observed in lot II; the magnitude, however, is smaller than lot I. These data also showed that water presoaking could not substitute for stratification, whereas, 12 to 24 hour presoaking with H_2O_2 might increase the germination speed to that of stratified seeds.

Effect on Respiration: The respiratory rate of air-dried seed was practically zero (0 hour in fig 2). As soon as water was available, the seed started to absorb water. The accumulated metabolic CO_2 and air of the intra- and inter-cellular space, and in inter-tissue space during storage were forced out by



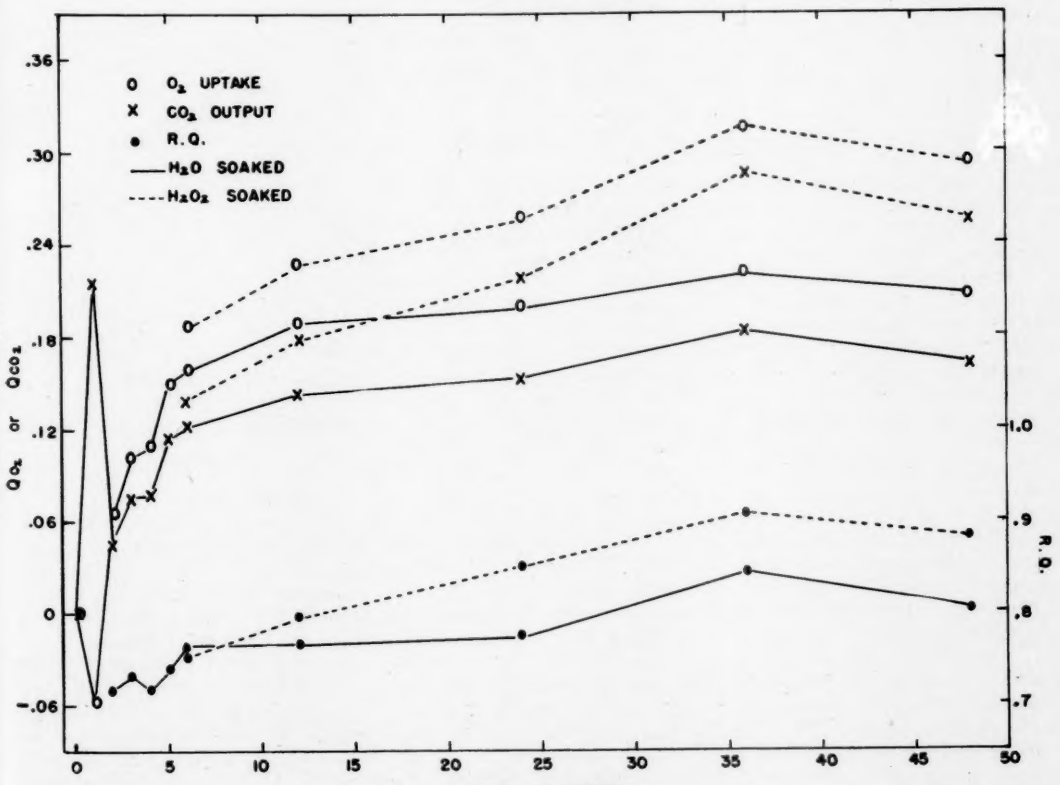


FIG. 1 (*top*). Accumulative germination percentages of lot I with different pretreatments. X——X dry seed, ○——○ stratified seed, ▲——▲ water soaked seed, ▲--▲ H_2O_2 soaked seeds, ... 50 % germination potential line.

FIG. 2 (*bottom*). Respiratory rate and respiratory quotient of water presoaked and H_2O_2 presoaked seed in lot I.

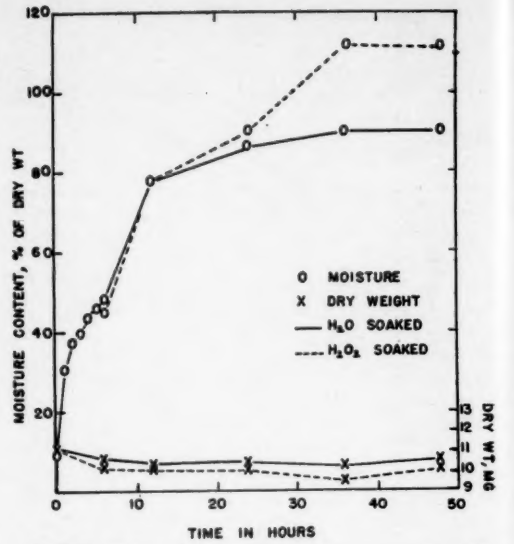
the swelling of cellular components. Thus production of O_2 and CO_2 was observed in the 1st hour after water was added to dry seed. The respiration started to increase in the 2nd hour, and reached a plateau at the 12th hour which coincided with the completion of imbibition (fig 3). At this point the seed contained 80 % moisture on a dry weight basis. Seed presoaked with H_2O_2 had a higher respiratory rate than water presoaked controls (fig 2). The respiratory quotients (R.Q.) of water presoaked seeds fluctuated between 0.65 and 0.85. Respiratory quotients of H_2O_2 presoaked seeds were definitely higher than those of water soaked seeds provided the presoaking time was 12 hours or longer in the 2 lots of seed.

Observations on the respiratory rate of seedlings at various stages of germination are summarized in figure 4. As can be seen, respiratory rate increased with the developmental stage of the seedlings. The seedlings produced from H_2O_2 presoaked seeds exhibited a higher respiratory rate than the control. Respiratory quotients showed a definite decline from 1.12 to 0.85 with seedling development in control, whereas seedlings from H_2O_2 presoaked seed possessed a somewhat lower R.Q. than that of control at all the stages. Seedlings with different pretreatment behaved similarly in lot II.

The average dry weights of seeds presoaked with H_2O_2 were less than those of water presoaked seeds in both lot I (fig 3) and lot II, while moisture contents of seedlings from H_2O_2 presoaked seed were in both cases slightly higher than those of the control (fig 4).

Discussion

A diagrammatic representation of the respiratory pattern and course of water uptake by germinating intact Douglas fir seed from lot I and lot II is shown in figures 5 and 6 respectively. The figures indicate 4 stages in the normal course of germination. The 1st, imbibition, is accomplished in 12 hours with almost a linear increase of respiration and water uptake. The 2nd stage, to 36 hours, is characterized by a constant respiratory rate and R.Q., and a temporary cessation of further water uptake. The 3rd stage prior to emergence consists of a gradual increase of water uptake and respiratory rate, and a rapid rise of R.Q. to approximately 1.15 at the time of radicle emergence (seedling stage A). The 4th stage consists of a further increase of respiratory and water uptake but with a decline of R.Q. in the seedling and the attached partially digested endosperm. The 1st stage is common to most seeds studied (11, 24, 25); the uptake of water probably creates the proper intracellular environment or a degree of hydration which is essential for enzyme systems to function (15, 22), and in the meantime the raised respiration probably supplies the energy requirement for the onset of the germination process (26). The 2nd stage has been observed by other workers at the stage prior to seed coat rupture (24). The low diffusion coefficient of O_2 (15) for a uniform rate of oxidation throughout



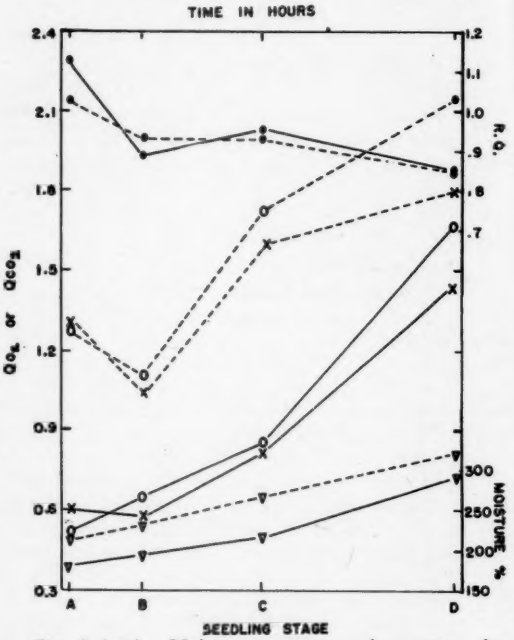


Fig. 3 (*top*). Moisture content and average dry weight of seeds with different pretreatment.

Fig. 4 (*bottom*). Respiratory rate, respiratory quotient and moisture content of young seedlings produced from seeds presoaked with H_2O and H_2O_2.

Legend:
- ○ O_2 uptake
- X CO_2 output
- • R.Q.
- △ Moisture content, % dry wt.
- — H_2O presoaked
- -- H_2O_2 presoaked

Seedling Stages:
- A–radicle 1 to 5 mm in length.
- B–radicle 6 to 10 mm in length.
- C–radicle 11 to 25 mm in length.
- D–radicle 30 to 40 mm and with epicotyl arch but no visible cotyledon expansion.

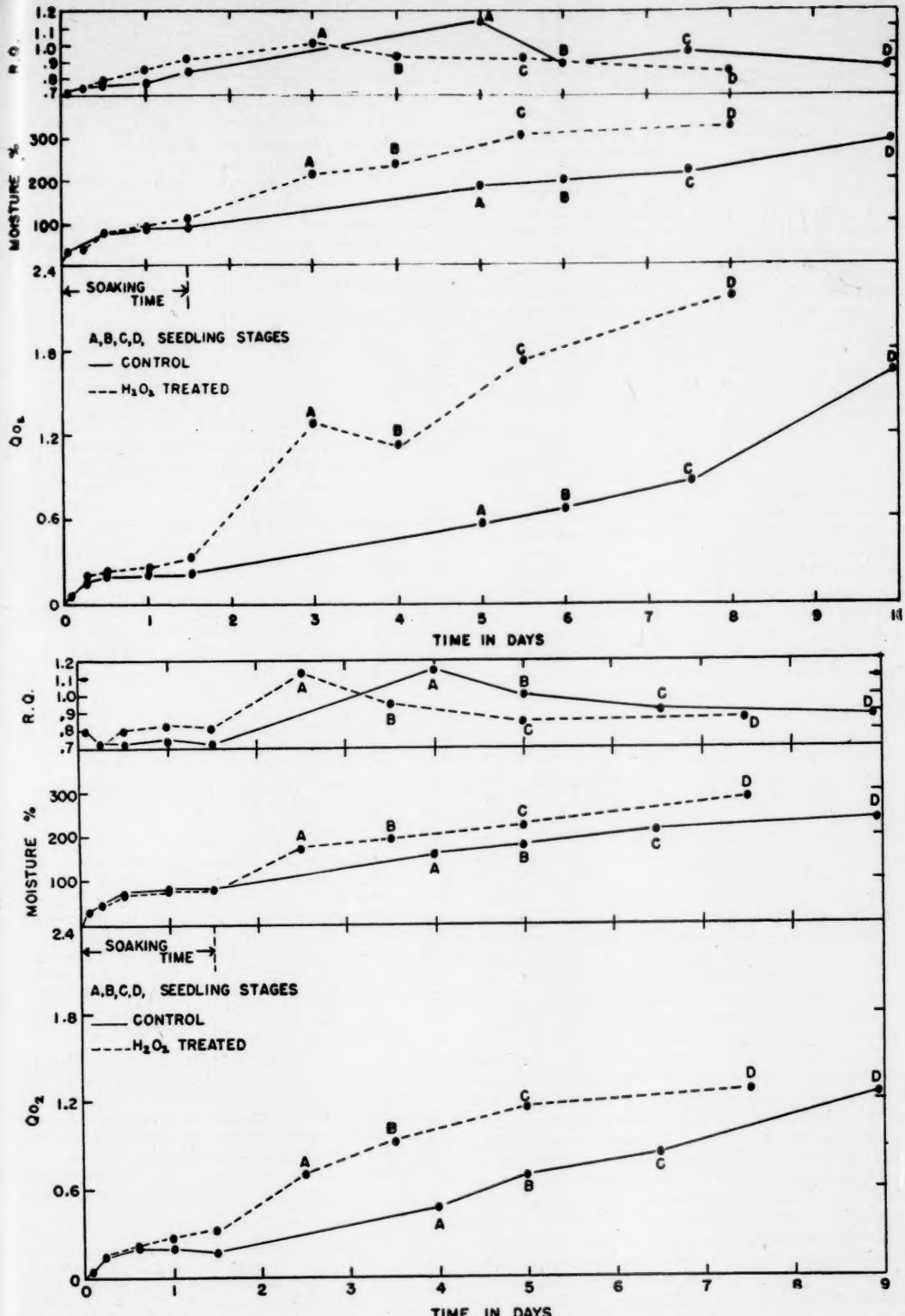


FIG. 5 (*top*). Diagrammatic pattern of respiration, moisture content, and respiratory quotient in the course of germination of Douglas fir seed (lot I).

FIG. 6 (*bottom*). Diagrammatic pattern of respiration, moisture content, and respiratory quotient in the course of germination of Douglas fir seed (lot II).

the tissue, the low concentration of essential substrates and cofactors for enzymatic reactions in catabolic as well as anabolic work, and/or the slow rate of enzymatic activities might be the limiting factors. It may be considered as an antephase of mobilization. The 3rd stage is the active mobilization of energy source and cellular components preparatory to the later stages of germination which are accompanied by true growth in terms of cell number, cell size and tissue differentiation. The 4th stage after radicle emergence symbolizes a remobilization for cotyledon emergence as evidenced by a lowering of R.Q. and increased respiration and water uptake. Experiments to study the chemical and biochemical changes during these stages would be desirable in order to further test this postulation. At present, however, it serves as a working hypothesis to be used for explaining the activation of H_2O_2 in germination.

Seed treated with H_2O_2 did not exhibit stage 2, that is, the constant rate of respiration and somewhat consistent R.Q., and the standstill of water uptake. The 3rd stage was considerably shortened, while the 4th stage proceeded with the same pace as the controls. The O_2 uptake and H_2O uptake were substantially higher in H_2O_2 treated seeds than those of the controls. These probably indicate that the rate of conversion of reserve fat to carbohydrates (9, 13, 18, 20) was higher, and more synthesis of cellular components (15) occurred in H_2O_2 treated seeds. The R.Q. was appreciably higher in H_2O_2 treated seeds prior to radicle emergence, while after the emergence, the R.Q. showed no significant difference from that of the controls.

It appears from these findings that H_2O_2 activates germination of Douglas fir seed by accelerating respiration during the antephase of mobilization. Several mechanisms possibly account for this activation. One, a direct destruction of the peroxide by catalase adds molecular oxygen which in turn increases the rate of respiration and so facilitates the oxidation of fatty substances (7, 15, 19). Two, a stimulation of lipoxidase by the chemical increases the oxidation rate of unsaturated fatty acids (4, 14). Three, a peroxidase functions with the H_2O_2 to oxidize growth inhibitors possibly present in tissues, e.g., high concentration of auxin (29). Four, the chemical acts directly as an electron acceptor when an appropriate hydrogen donor is present (6, 10, 15, 19); thus the rate of respiration rises.

The oxidative phosphorylation coupled with respiration and degradation of fatty substances conserves energy (e.g., as adenosine triphosphate, ATP) which activates many of the enzyme systems and synthetic reactions (5, 7, 19) which are essential for mobilization of cellular components and energy for synthetic reactions involved in growth. Marré and Forti (16) found that the auxin induced increase of respiration results in a rise of ATP level in tissue; thus this postulation does seem plausible. Shifting equilibria of other key reactions in the germination process by H_2O_2 is possible, the present study is too limited to reveal such a possibility.

Summary

Stimulation of Douglas fir seed germination was obtained by soaking seeds in 1 % H_2O_2 for 36 to 48 hours at room temperature. The H_2O_2 presoaked seeds and seedlings from the treated seed exhibited a marked increase of respiratory rate and respiratory quotient over those water soaked controls. Fresh seeds collected at a high elevation responded to H_2O_2 activation more dramatically than the seeds collected at a lower elevation and subsequently stored at freezing temperature, although the mode of action was similar.

The respiratory pattern, course of water uptake, and R.Q. change during germination of intact Douglas fir seed were described. Possible mechanisms of stimulation by H_2O_2 treatment were discussed.

Note added in proof: Dr. P. K. Stumpf reported at the Symposium on Foods—Enzymes, held on September 9 to 10, 1959, Oregon State College, that they recently found the 2-oxidation of fatty acid is enhanced by hydrogen peroxide via the intermediate of aldehyde and with a release of carbon dioxide and fatty acid of shorter chain. This discovery might explain the mechanism of activation reported herein.

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ALTERATION OF THE PROTEIN COMPONENTS AND OTHER CONSTITUENTS IN THE TOBACCO LEAF BY SHADING[1]

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Leaf proteins, like most of those of animal origin, are in a continual state of flux (19). Turnover phenomena have been found in leaves (31), and the concentration of protein is markedly influenced by the physiological age of the leaf (11) as well as by environmental factors such as temperature (4), supply of moisture (23), and nutritional status (25). Even diurnal fluctuations in the level of protein have been claimed to occur in leaves (27).

Chibnall and Grover (6) first demonstrated that leaf proteins could be fractionated into 2 major groups, 1 soluble and the other insoluble in aqueous salt solutions. Since then, electrophoretic (10, 29, 33) and ultracentrifugal studies (24, 26), investigation of the incorporation of N^{15}-labeled ammonia into various soluble and insoluble fractions (8), and the purification or crystallization of numerous enzymes from leaves have provided ample physiological and chemical demonstration of a wide array of individual protein components in the leaf.

However, most studies of the factors which affect these important constituents have not distinguished between the various fractions, but have dealt with the aggregate of proteinaceous material in the leaf. Nevertheless, evidence, derived mainly as a by-product of studies of plant viruses, indicates that all of the normal protein components do not respond alike to shifts in internal and external conditions. For instance, fractionation of leaf proteins by electrophoresis (2), by ultracentrifugation (9), and by separation of the protein into a soluble and an insoluble fraction (12) has shown that change in the physiological age of the leaf does not affect every group of proteins studied in the same way. Measurements of pronounced change in the activity of enzymes from leaves grown under different environments (20, 30) suggest that individual protein components also differ

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widely in their response to external conditions. This has been demonstrated directly by Lyttleton (17, 18) who showed that at least part of the high molecular weight fraction of the soluble leaf protein accumulates only in the light.

In many plant species, expansion of leaves is more vigorous under reduced light intensities than in direct sunlight. Changes in composition of the leaf also occur when the intensity of light received by the plant is altered. While studying biochemical effects produced by shading tobacco leaves, it was noted that the proteins, particularly, underwent marked changes. Zone electrophoresis revealed that major quantitative differences existed between the soluble protein fractions of leaves grown either in a humid, shaded environment, or under drier conditions in full sunlight. These results are reported here as a demonstration that experimentally imposed environmental conditions can alter selectively the quantity of a specific fraction of the soluble protein in the leaf. The relationship of the change in protein constituents to the morphological and biochemical differences observed between the sun and shade leaves is also discussed. However, since electrophoresis yields only gross, inhomogeneous groupings of the many individual leaf proteins, no specific effects can yet be assigned to these alterations.

Materials and Methods

Shade leaves refer to leaves of *Nicotiana tabacum*, var. Connecticut 49 obtained from plants grown outdoors under a cheesecloth shade tent during the seasons of 1955 to 1958. Sun leaves were obtained from similar plants except that the seedlings were transplanted to an unshaded plot adjacent to the shade tent. Measurements have shown that the cheese cloth reduced the light intensity, which can exceed 10,000 ft-c, by an average value of 20 to 30 % and promoted a more humid environment than that of the open field, but did not alter the soil or air temperature. Plants in both plots were amply supplied with organic and inorganic forms of nitrogen throughout the growing season. All comparisons were made between triplicate samples of 6 to 10 leaves excised from corresponding nodes of sun and shade plants, 1 leaf being taken from each plant. Only laminar tissue of the leaves was used for analysis. Since sun plants did not differ from shade plants in their general morphological development (they produced the same number of leaves and flowered at the same time as shade plants), this method of collection provided physiological and anatomically comparable samples of the 2 leaf types.

Protein nitrogen (alcohol insoluble N) was determined by micro-Kjeldahl digestion of the nitrogenous components insoluble in cold, 70 % alcohol, followed by Nesslerization of the NH_3 produced. Separation of leaf proteins into a soluble and an insoluble fraction was accomplished by grinding the tissue in a cold glass homogenizer with 5 to 10 volumes of chilled 0.1 M potassium phosphate buffer at pH 7.0 and centrifuging the homogenate for 20 minutes in the cold at 20,000 × G. The precipitate, after being washed with buffer, contained the insoluble proteins while the combined clear supernatant fluid and washings contains the soluble proteins.

The endogenous O_2 consumption of 8 mm leaf disks was measured at 30° C in a Warburg respirometer over a period of several hours.

Assays of enzymes were made on cell-free extracts prepared from mature sun and shade leaves according to the method of Zelitch and Zucker (35). The assays of glycolic acid oxidase (35), malic dehydrogenase (22), and glyoxylic acid reductase (34) were carried out in duplicate at several concentrations.

Zone electrophoresis was performed on purified potato starch (14) which had been washed with distilled water and air dried. Protein extracts used for electrophoresis were prepared by grinding leaf disks in 25 ml of cold 0.05 M potassium phosphate buffer at pH 6.9 which contained cysteine hydrochloride at 0.002 M. The cysteine was added to prevent formation of highly polymerized phenolic pigments (13). Each sample of disks, taken according to a fixed arbitrary pattern from the central part of 6 to 10 leaves, represented an area of 170 sq cm and weighed about 3.5 g. The extracts, after being clarified by centrifugation at 20,000 × G for 20 minutes, were brought to 80 % of saturation with ammonium sulfate. The precipitate was collected and dissolved in 3 ml of cold 0.012 M phosphate buffer at pH 6.9 (no added cysteine) and the solution was dialyzed 24 hours in the cold against a large volume of this buffer. Prior to electrophoresis, the dialyzed solution was clarified by centrifugation. The starch strips were formed in a mold consisting of a lucite frame supported on glass and divided into 2 compartments, each 2.5 × 35 cm. A quantity of 50 g of starch was 1st washed and then suspended in the 0.012 M phosphate buffer (ionic strength, 0.025) and poured into the frame. After the excess of moisture which escaped from the bottom of the frame was blotted from the starch, the frame was removed and a transverse slice 5 mm wide was cut 10 cm from 1 end of each strip and replaced by approximately 0.5 ml of protein extract which had been mixed into a paste with dry starch. Extracts from sun and shade leaves were compared side by side. The strips were connected to buffer chambers containing the 0.012 M buffer at pH 6.9 by means of 2 filter paper wicks and a potential gradient of 350 V was applied for 6 to 7 hours at 4° C. The current ranged from 2 to 4 m. amp. After electrophoresis, the strips were sliced transversely into 5 mm sections, and each section was eluted with 2 ml of cold water for at least 2 hours. The absorbance of each eluate was determined at 280 and 260 $m\mu$ by means of a Beckman DU spectrophotometer. Protein was also measured in aliquots of the eluates by the method of Lowry et al (16). Both methods of determination gave similar electrophoretic patterns.

The concentration of reducing sugars was measured colorimetrically in alcoholic extracts of leaves (21).

Results

GROSS EFFECTS OF SUN AND SHADE ENVIRONMENTS: Plants in the open field increased in height more slowly than shaded plants (see Stewart (28)), and shade leaves reached a considerable size before sun leaves in the same node position began to expand rapidly. The customary thickening of the leaf usually associated with a sunny environment was not observed by either macroscopic or microscopic examination.

Several characteristic differences in composition of sun and shade leaves are shown in table I. Initially, sun leaves accumulated less dry matter than shade leaves of the same age although, by maturity, they equalled or surpassed shade leaves in dry weight (table I). The concentration of reducing sugars was much higher in sun leaves throughout development, but very little starch accumulated in either type or leaf. Sun leaves were also distinguished from shade leaves by a higher level of total protein nitrogen. When expressed on a per leaf basis, the protein N of sun leaves (table II) followed the usual pattern of accumulation during rapid expansion and loss during maturation and senescence. On the other hand, the protein content of shade leaves reached a maximum at an earlier age than the corresponding sun leaves, indicating that they had developed more rapidly.

TABLE I

EFFECT OF SHADING ON GROWTH AND COMPOSITION OF TOBACCO LEAVES

TYPE OF LEAF*	AREA	DRY WEIGHT	REDUCING SUGARS, AS GLUCOSE	TOTAL PROTEIN N
4th node from base of plant	*sq cm per leaf*	*g per leaf*	*μg per sq cm*	*μg per sq cm*
Young shade leaf	627	1.41	58	108
" sun "	388	.94	81	117
Mature shade leaf	834	1.73	17	55
" sun "	716	1.84	30	78
13th node from base of plant				
Young shade leaf	842	1.64	73	97
" sun "	424	1.07	152	129
Mature shade leaf	1143	2.25	75	60
" sun "	1047	2.51	92	72

* Sun and shade leaves in each pair of samples were of the same numerical age.

ENZYME STUDIES: When activities of soluble enzymes were determined in extracts of mature leaves, considerable differences were found between the 2 leaf types. The activity of glycolic acid oxidase, malic dehydrogenase, and glyoxylic acid reductase in extracts of sun leaves was almost twice that in extracts of shade leaves (table III). The soluble protein nitrogen was also nearly doubled. Consequently, the specific activities of these enzymes did not vary greatly between the 2 types of leaves. However, glyoxylic acid reductase did show a significantly higher specific activity in extracts of sun leaves.

TABLE II

TOTAL CONTENT OF PROTEIN NITROGEN IN SUN AND SHADE LEAVES OF VARYING AGES

AGE OF LEAF FROM SEEDLING STAGE		PROTEIN N	
		SUN	SHADE
	days	*mg per leaf*	
4th Leaf	34	44.6	67.6
	43	64.0	65.4
	55	59.2	46.1
13th Leaf	49	55.6	79.1
	64	91.2	79.0
	77	75.5	68.5

The increased activity of soluble enzymes from the sun leaves was accompanied by a higher rate of endogenous O_2 consumption per sq cm of whole leaf tissues (last column, table III). When expressed on the more common basis of dry weight, the O_2 consumption of sun leaves still indicates that they had a more active metabolism than shade leaves, the actual values being 1.84 and 1.58 μl of O_2 per hour per mg of dry weight respectively.

The large differences noted above in the concentration of soluble proteins suggest that sun leaves contained a disproportionate amount of these components. The data in table IV demonstrate that the ratio of soluble to insoluble protein is indeed greater in sun leaves, and that most of the difference in the total protein N of the 2 leaf types is attributable to the soluble protein fractions.

ZONE ELECTROPHORESIS OF THE SOLUBLE PROTEINS OF THE LEAF: Electrophoresis on starch substantiated the indications given above of quantitative differences in the composition of the soluble protein fraction from the 2 types of leaves. Electrophoretic patterns of soluble proteins from corresponding young, actively growing sun and shade leaves are presented in figures 1 to 3. Extracts of young shade leaves gave patterns closely resembling those of Takahashi and co-workers (10, 29) and of Bawden and Kleczkowski (2) obtained by conventional moving boundary electrophoresis. A single major peak with faster and slower moving shoulder components, along with a fraction that did not move from the origin, was observed repeatedly in extracts of expanding shade leaves. Extracts of corresponding sun leaves showed a strikingly different pattern in respect to the ratio of components in the major peak to those in the faster moving shoulder. Indeed, this latter fraction, a minor one in shade leaves, increased to such an extent that in half-expanded sun leaves (figs 2 and 3) it became the major peak. No extracts of shade leaves, regardless of age, were observed to contain more than 1 major electrophoretic fraction, whereas all extracts from young, rapidly growing sun leaves showed 2 distinct peaks, the faster moving fraction being at

times greater in area than the major peak of slower mobility. Consequently, the observed difference between extracts from sun and shade leaves could not be merely a reflection of disparity in physiological age of corresponding leaves. This change from a minor fraction in the shade leaf to a major component in the sun leaf was the only consistently detected difference in electrophoretic patterns of the soluble proteins of 2 leaf types. Unfortunately, the electrophoretic method used did not admit of quantitative application of protein solutions to the starch strips. Therefore, it was not possible to determine whether all or only part of the difference in the concentration of soluble proteins between sun and shade leaves was accounted for by the increased quantity of the faster moving fraction in the sun leaf.

Electrophoretic patterns of the proteins of mature leaves (figs 4 and 5) were less complex than those of young leaves. The extracts from mature leaves showed only a single major peak and a small fraction at or near the origin. These patterns were similar to diagrams in the literature (7) obtained by conventional techniques, and they differed from patterns of proteins from young leaves in the manner described previously by Bawden and Kleczkowski (2). Obviously, the differences in electrophoretic mobility between the major protein fractions of rapidly growing sun and shade leaves are no longer found in the soluble proteins of fully expanded mature leaves. Only the gross difference in the total amount of soluble protein remains (see table III).

In addition to the fractions mentioned above, a positively charged component similar to the one reported by Takahashi and Ishii (29) was observed in extracts of both young (fig 1) and old leaves. In some electrophoretic runs, a fast moving fraction was detected (fig 3) which corresponded roughly in mobility and absorption of ultra-violet light to the free nucleic acid component of Linder, Kirkpatrick and Weeks (15) and of Bawden and Kleczkowski (2).

Visual observation of the starch strips during electrophoresis showed that a number of pigments were present. The major protein fraction occurring in both types of leaves as well as the 2nd peak in sun leaf extracts always contained yellow-brown pigments. Consequently, extracts of young sun leaves were easily recognized on the starch strips, for they contained 2 quite distinct moving bands. The fraction at the origin was associated with a yellow pigment. However, the intensity of the pigment in this band was not correlated with the amount of material which absorbs at 280 mμ. Occasionally, ultra-violet light revealed a yellow fluorescent band near the origin. The major peak of the extracts of shade leaves and the 2 major fractions from young sun leaves usually had ratios of absorption at 280 and 260 mμ greater than 1, varying from 1.1 to 1.3. In some experiments, enough material was collected from the peak fractions to confirm their protein nature by positive biuret reactions.

TABLE IV

PARTITION OF PROTEIN BETWEEN THE SOLUBLE AND INSOLUBLE FRACTION OF SUN AND SHADE LEAVES

LEAF TYPE	LEAF SIZE	SOLUBLE PROTEIN NITROGEN	INSOLUBLE PROTEIN NITROGEN	TOTAL PROTEIN NITROGEN
	% of final leaf area	*μg of protein N per sq cm*		
Shade	46	58.0	32.0	90.0
Sun	49	73.3	34.4	107.7
Shade	78	57.0	36.0	93.0
Sun	85	77.5	42.2	119.7

DISCUSSION

The experimentally imposed conditions of growth employed here have selectively altered a specific electrophoretic fraction of the soluble protein from the tobacco leaf. Such experimental control over the accumulation of specific fractions of the normal soluble protein opens the way for investigations into both their composition and their possible functional significance.

Differences in composition and in growth rates observed between the 2 leaf types studied here are characteristic of those distinguishing sun and shade leaves of many species. The differences in environment which produced changes in the tobacco leaves

TABLE III

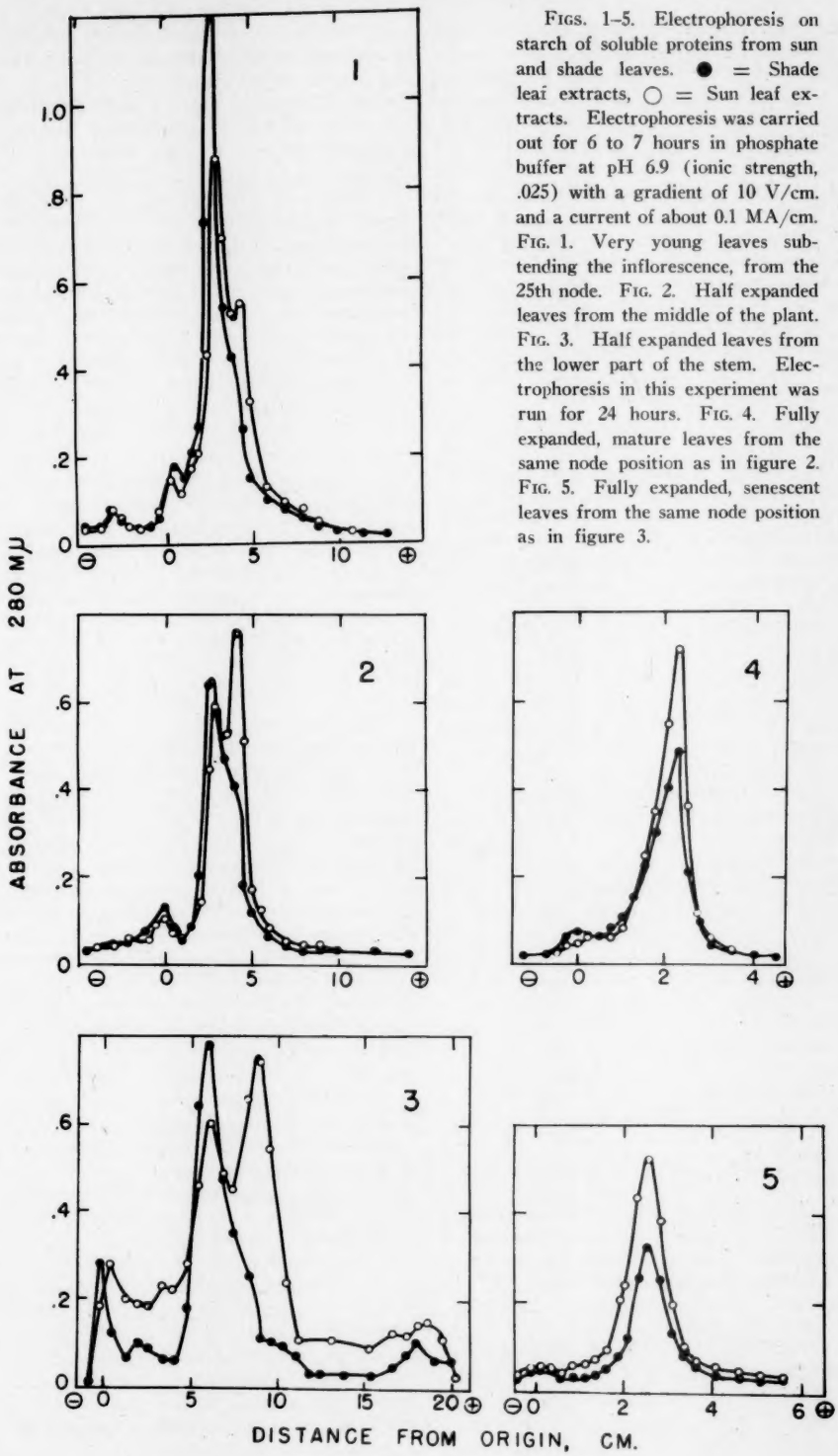
OXYGEN CONSUMPTION OF LEAF DISKS AND ACTIVITY OF SOLUBLE ENZYMES IN EXTRACTS OF SUN AND SHADE LEAVES

TYPE OF LEAF EXTRACTED	FINAL VOLUME OF EXTRACT	PROTEIN N PER ML OF EXTRACT	GLYCOLIC ACID OXIDASE		MALIC DEHYDROGENASE		GLYOXYLIC ACID REDUCTASE		OXYGEN UPTAKE
			UNITS**	SA*	UNITS***	SA	UNITS***	SA	
	ml	*mg*	*per ml*		*per ml*		*per ml*		*μl per hr per sq cm*
Shade (5th node)	400	1.63	11.6	7.11	450	275	33.8	20.7	4.84
Sun (5th node)	400	2.75	20.9	7.61	860	306	80.8	29.4	6.86

* SA signifies specific activity of the enzyme in units per mg of protein N.

** One unit of enzyme is the amount necessary to cause the uptake of 1 μl of O_2 per 10 min at 30° C.

*** One unit of enzyme is the amount necessary to cause a decrease in absorbance at 340 mμ of 0.01 per min.



FIGS. 1–5. Electrophoresis on starch of soluble proteins from sun and shade leaves. ● = Shade leaf extracts, ○ = Sun leaf extracts. Electrophoresis was carried out for 6 to 7 hours in phosphate buffer at pH 6.9 (ionic strength, .025) with a gradient of 10 V/cm. and a current of about 0.1 MA/cm. FIG. 1. Very young leaves subtending the inflorescence, from the 25th node. FIG. 2. Half expanded leaves from the middle of the plant. FIG. 3. Half expanded leaves from the lower part of the stem. Electrophoresis in this experiment was run for 24 hours. FIG. 4. Fully expanded, mature leaves from the same node position as in figure 2. FIG. 5. Fully expanded, senescent leaves from the same node position as in figure 3.

involve alterations in the water relations of the plant as well as differences in light intensity (see Materials and Methods). Both factors influence the proteins of the leaf. Drought, similar to the sun environment, increases the total protein concentration of the leaf and the activity of some soluble enzymes (30). Hanson (12) has shown that withholding water will also increase the ratio of soluble to insoluble proteins in the leaf. However, this effect resulted from a rapid loss of insoluble protein rather than from an accumulation of soluble protein such as that which occurred in the sun leaves studied here. Changes in light intensity have been shown also to alter the protein composition of the leaf (17). Consequently, the specific factors of the environment which may produce an accumulation of the faster moving major fraction in young sun leaves remain to be identified.

That the difference in electrophoretic patterns between the 2 types of plants is found only in extracts of young leaves emphasizes the point that the effects observed may reflect an interference with the early development of the leaf. This substantiates the morphological observations that the most striking visual differences between sun and shade plants occur during their early growth. Once rapid expansion has begun, sun leaves have a more active metabolism than shade leaves as indicated by the data on differences in composition, O_2 consumption and enzymic activity. This is further supported by the fact that sun leaves of many species have a greater maximum rate of photosynthesis (3, 32). Yet, the apparent increase in metabolic activity does not lead to a larger, more vigorously growing plant.

Bawden and Kleczkowski (2) reported that a minor soluble protein fraction from tobacco leaves, similar to the one which accumulated in young sun leaves, increases during nitrogen deficiency, but no data were given to indicate the magnitude of the effect. Since, in the present work, sun leaves were found to contain a higher concentration of protein than shade leaves, the changes in electrophoretic patterns described cannot be attributed to such a deficiency. On the other hand, both the lack of nitrogen and exposure to full sunlight inhibit the growth of the leaf. Consequently, the accumulation of the faster moving fraction by young sun leaves may represent a response to the inhibition of growth rather than a specific effect of the environment on the protein metabolism in the leaf.

The general decline in the level of protein which begins in the shade leaf when rapid growth ceases could practically eliminate certain of the minor fractions originally present in young leaves. Thus, electrophoretic patterns of proteins from mature shade leaves might be expected to contain only the single major component found in the young leaf. However, since young sun leaves contain not 1 but 2 major soluble protein fractions, electrophoretic diagrams of extracts from mature sun leaves might be expected to contain an additional component not present in corresponding shade leaves. This is not the case. Therefore, the 2nd major component in sun leaves must be much more labile (either differing in rate of synthesis or of breakdown) than the slower moving fraction found in both types of leaves. This apparent difference in stability of the 2 protein fractions correlates well with the findings that enzymes, as measured by their activities, show a wide range of stabilities in excised tobacco leaves. (1, 35).

Because of the relatively few fractions obtained on electrophoresis of the innumerable soluble proteins, no fraction could be considered homogeneous. The activity of a number of enzymes such as glycolic acid oxidase (unpublished data of I. Zelitch) and nitrate reductase (5) increase with leaf age. Yet, no fractions have been detected which show similar trends. It is also surprising that the proteins of the narrow strap-shaped leaves which subtend the inflorescence should display the same electrophoretic pattern (fig 1) as those from much larger, morphologically different leaves from the middle of the stem (fig 2). This again may be a reflection of the small extent of fractionation obtained by electrophoresis. The separation of sun leaf proteins into 2 major electrophoretic groups raises the question of biochemical affinities among the proteins in each group. Further fractionation and study of enzymic activities associated with the individual components from each peak may reveal interesting relationships.

Summary

Studies of the protein and other constituents in leaves from sun and shade grown tobacco plants revealed distinct differences in composition. Initially, sun leaves developed more slowly biochemically and morphologically than shade leaves, but, when fully grown, sun leaves showed a more active metabolism as measured by accumulation of dry weight, reducing sugars, and protein and by the endogenous rate of O_2 consumption. The concentration of certain soluble enzymes in mature sun leaves was almost twice that found in mature shade leaves. However, little difference in specific activity was found, for the concentration of soluble proteins in sun leaves was also correspondingly greater. The disparity in concentration of insoluble leaf proteins was much less. Consequently, sun leaves also had a higher ratio of soluble to insoluble proteins than shade leaves. Indeed, most of the difference in total protein nitrogen could be accounted for by the difference between the soluble fractions of the 2 leaf types.

Electrophoresis on starch of the soluble proteins revealed further differences in composition. Young shade leaves exhibited only 1 major soluble fraction, whereas young sun leaves contained an additional major peak corresponding in mobility to minor shoulder components in the shade leaf extracts. No difference between the electrophoretic patterns of soluble proteins from mature sun and shade leaves was observed. The findings demonstrate that specific protein fractions in tobacco leaves can be altered selectively by experimentally imposed conditions of growth.

I would like to thank Dr. I. Zelitch and Mrs. Marcia Sutter for their extensive aid in the enzyme studies.

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EXPERIMENTAL MODIFICATION OF PLANT SENESCENCE[1, 2]

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The termination of the life cycle of annual and biennial plants is sudden and complete. Entire fields of grains and other annual crops die simultaneously, often in the early part of the growing season. Neither the function nor the physiological basis for this senescence is understood.

Plant senescence has received little attention since the publication of a small, perceptive book by Molisch (6) 30 years ago. He noted that plants which were delayed in the completion of the reproductive stages would show delayed senescence. For example, century plant (*Agave americana* L.) has a life span of 8 years in some climates and 100 years in others depending upon how soon it becomes reproductive; whenever it flowers and fruits the plant dies. He carried out experiments with various species of annual plants, and found that removal of flowers effectively extended the life span. From these observations he deduced that ". . . when we hinder flower formation . . . we guard the plant against death . . . but just as soon as it has flowered and fruited, the organic reserves are completely emptied and utilized and a yellowing of the entire plant accompanies this process . . . and for this reason the plant dies after ripening of its seeds."

The present experiments provide a quantitative extension of these observations of Molisch, and provide evidence that the deduced cause of senescence is more complex than a depletion of food reserves.

METHODS

Experiments with soybean (*Glycine max* Merr. var. Lincoln) were carried out in the field with 10-foot rows planted on May 12, 1956. After the first appearance of flowers defloration was carried out 3 times per week on triplicate rows. Flowers were removed as soon as they showed petal color. The experiment was terminated by a killing frost on October 10. Similar experiments were carried out in the summers of 1957 and 1958. More detailed experiments were carried out in the greenhouse in 1958, using various times of flower and fruit removal.

Experiments with spinach (*Spinacia oleracea* L. var. Long Standing Bloomsdale) were carried out in the greenhouse. Plants were selected from large populations for uniformity. In a typical experiment about 60 % of the plants were discarded in order to attain maximal uniformity in the experimental material—an essential if reliable measurements of small differences in senescence rates are to be obtained. At the beginning of the experimental period all plants were decapitated in the internode above the 10th flowering node. At the same time, leaves from the basal rosette were removed in order that the quantitative measurements of senescence should start from zero. As an additional step to increase the precision

FIG. 1. Delay in senescence of soybean plants by defloration (left) as compared to control plants (right). Photographed September 7, 1956.

FIG. 2. Delayed senescence in soybean plants by various treatments: (a) flowers removed as petals show color, (b) fruits removed after pods had elongated but not filled, (c) fruits removed at first sign of yellowing with ripening, and (d) untreated controls.

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of the experiments, only plants at an identical stage in flowering were utilized in comparable groups.

Senescence was followed quantitatively as evidenced by the degeneration of the leaves. Each leaf on each plant was scored by an arbitrary scale as follows: 0) leaves entirely green, 1) leaf tips necrotic, 2) leaf tip and peripheral area dead, 3) half of leaf dead, and 4) entire leaf dead. Scoring of plants was repeated 3 times per week. Each treatment was applied to 6 to 12 plants carefully selected for uniformity. Lateral branches were removed as soon as they appeared. Each test reported was repeated with parallel results.

Experimental

Soybean plants were deflorated as petals showed color or defruited as the pods became nearly filled but before the yellowing color change of the fruit associated with ripening. These treatments completely prevented the senescence of the plants within the growing season. The appearance of the deflorated plants and the untreated controls is illustrated in figure 1, where it will be seen that all plants in the treated group were green and healthy whereas all plants in the fruiting controls were completely dead. This experiment has been repeated for 3 summers. The same complete prevention of senescence is obtained when the fruits are removed, though there is a somewhat more complete leaf retention in the deflorated group than in the defruited group.

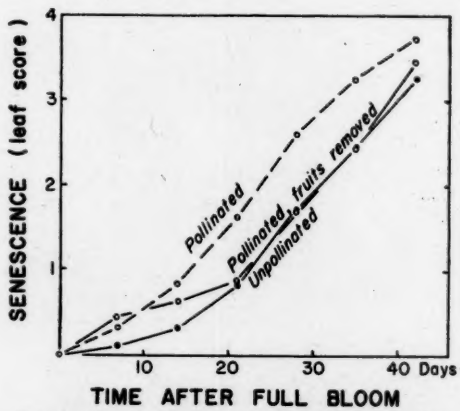
The induction of senescence by the reproductive activities of the plant may be related to any of a number of stages in the flowering and fruiting processes. This is readily seen from greenhouse experiments with soybean plants which were (a) deflorated at the time of 1st petal color, (b) defruited after pods had elongated but not filled out, or (c) defruited after the fruits were entirely filled and just starting to show the yellowing color change associated with ripening. Control plants died at an average of 119 days after sowing, and defruited and deflorated plants were increasingly persistent. The appearance of representative plants 119 days after sowing can be seen in figure 2. The deflorated plants are the most vigorous, the early defruited and late defruited were less vigorous, and the untreated plants were entirely dead. The average time of plant death was recorded for the treatments as follows: Deflorated, 179 days; defruited when pod full length, 161 days; defruited when pod filled, 129 days; and control, 119 days.

To permit a more precise analysis of the effects of various stages of reproduction, experiments were carried out with dioecious spinach plants. Defloration and defruiting treatments were carried out on staminate and pistillate plants at different stages of development.

In the spinach it was soon found that once seed stalk development or bolting has begun, senescence will surely follow and the removal of flowers or fruits can only delay this approaching demise. The comparative effects of different treatments on senescence can be estimated by the extent to which senescence is delayed.

The defruiting of pistillate spinach plants was effective in delaying senescence. Effects of this treatment are shown in figure 3. A useful index in comparing the rates of senescence of the various treatments is the time required for the plants to reach an intermediate stage of senescence. For example, pollinated plants reached a senescence score of 2.0 after 25 days, whereas plants from which the fruits were removed or which remained unpollinated reached this value after 31 days. In the latter case the unpollinated ovaries enlarged but never matured into ripe fruits.

In this experiment fruits were removed from some plants on the 5th day after pollination and it can be



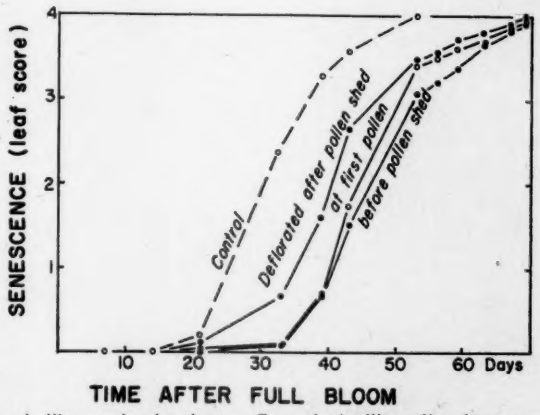


Fig. 3. The development of senescence with time in pistillate spinach plants. Control (pollinated) plants are compared with plants defruited 5 days after pollination and unpollinated plants.

Fig. 4. The development of senescence with time in staminate spinach plants. Control plants are compared with plants deflorated after pollen shed (day 10), at the time of first pollen shed (day 1) or before pollen shed (day 0).

seen that after day 5 the senescence score rapidly became essentially identical with that of the unpollinated controls. In other experiments the removal of fruits was carried out 10 and 20 days after pollination, and in each case the senescence score became essentially the same as that of the unpollinated plants within 10 days after defruiting. Senescence curves of unpollinated plants were not significantly different from those of deflorated plants.

Senescence of staminate spinach plants was likewise delayed by the removal of the flowers. In a sample experiment flowers were removed as they first became identifiable, as dehiscence of the 1st anther occurred, or after the dehiscence of all anthers. The senescence of the plants receiving these 3 treatments is compared in figure 4. It will be seen that the average senescence score for the plants which were not deflorated rose relatively rapidly, and the onset of senescence was delayed increasingly with earlier and earlier defloration. The differences in senescence of plants deflorated when the sex of the flowers became first detectable and when the 1st anther dehisced is probably not real.

The appearance of staminate deflorated plants after 45 days as compared to controls is shown in figure 5. The control plants were dead and the deflorated plants still green and erect.

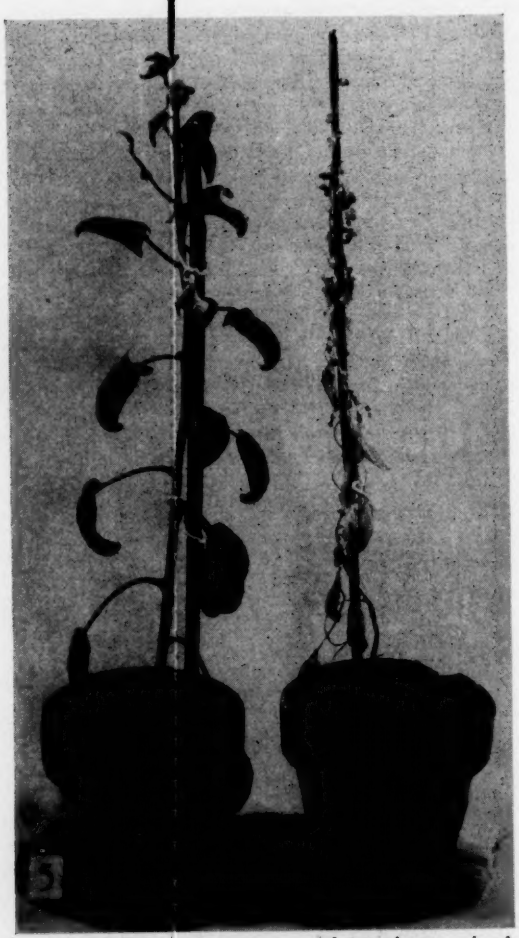


FIG. 5. Delay in senescence of staminate spinach plants by defloration (left) as compared to control plants (right). Photographed 45 days after full bloom.

DISCUSSION

Senescence appears to be imposed on a plant by an increasing signal, intensified with successive stages in the reproductive development of the plant. In spinach, the induction of flower initiation and bolting appears to start such a signal, for if plants are kept in short photoperiods which maintain a vegetative rosette condition they appear to have a quality approaching immortality, maintaining a healthy green condition for an indefinite period of time. All plants die within a period of 11 weeks after bolting. An intensification of the signal to senescence is associated with the shedding of pollen and, to a larger extent, the degeneration of the staminate flower parts. This is clearly seen in the staminate spinach plants which are delayed in senescence by defloration. A further intensification is associated with the time of fruit enlargement, as indicated by the pistillate spinach plants which are delayed in senescence if the fruits are removed at various times after pollination. And a final increase occurs with the commencement of ripening of the fruits in the bean plants, as indicated by the marked delay in senescence if the fruits are removed shortly before the color changes associated with ripening.

If the concept of Molisch were a complete explanation of the onset of senescence and the organic reserves were emptied from the plant into the developing fruits, then there would be no senescence in the male plants and the delay in senescence in soybeans following the removal of the filled but not yet ripened fruits would not be expected. An interesting side effect of the defloration and early defruiting treatments was an increase in flowering which followed. In fact the defruiting treatment resulted in a considerable increase in the numbers of fruits which were filled and then removed. Thus if the depletion theory were the explanation of senescence then the defruiting treatment might logically have been expected to hasten rather than retard senescence. Surely the depletion effect is involved and contributes to senescence, but we feel that it is only a partial explanation of the onset of death.

It has long been known that flowering and fruiting cause a retardation of growth (7), and it follows that the more vigorous the flowering the more rapid the senescence (11). Many effects have been observed to follow the removal of flowers. In addition to the deferred senescence (6) there is an increase in growth rate (7), reduction in flower abscission (13),

an increase in subsequent fruit set (8), and an extension of the duration of flowering (5).

There have been 3 general types of explanations proposed to account for senescence. (a) It has been attributed to excessive water loss associated with the time of flowering (1). This theory has never been seriously considered, though it is clear that wilting of leaves can certainly induce leaf senescence (4). (b) The suggestion has often been made that a decline in available nutrients could cause senescence—the general concept of Molisch. Thus aging leaves show a more rapid utilization of carbohydrates (14, 16), more export of inorganic materials (2, 15), and are less effective in photosynthesis (3, 12). (c) Murneek has proposed that the developing embryos induce senescence in the parent plant. This has been explained as a competition by the embryos for food reserves (9) or, more recently, as a possible production of inhibitors by the developing embryos (10).

While these various physiological events may certainly be contributing to the development of senescence, the evidence provided here cannot be explained on the basis of any or all of them. These experiments suggest an accumulation of influences inducing senescence with each of the developmental stages of reproduction in soybeans and spinach. Establishing a quantitative basis for the measurement of these influences should facilitate the physiological definition of their nature.

Summary

The onset of senescence in soybeans and in spinach plants has been effectively delayed by the removal of either flowers or fruits. The relative effectiveness of such treatments declines progressively as the time of removal is delayed, both in soybeans and in staminate and pistillate plants of spinach. The experiments support a concept of senescence in response to a gradually intensifying signal in reproductive plants, rather than to a depletion of food reserves through mobilization into developing fruits.

Grateful acknowledgement is made to Dr. S. L. Lam for carrying out the greenhouse experiment with soybeans.

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LATITUDINAL EFFECT ON RESPIRATION IN SOME NORTHERN PLANTS[1,2]

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Comparative measurements have been made on a great many species of cold-blooded animals from different latitudes, and generally it has been found that the aquatic forms that live in the Arctic respire at a higher rate for a given temperature than those from more southern latitudes. Among terrestrial insects, however, such a metabolic compensation either has not been found or is very much less pronounced, indicating perhaps that insects can to some extent select favorable microclimates (1, 9). That at least some of them are capable of compensating has been demonstrated in the case of cockroaches by keeping them for prolonged periods at low temperatures (3). In plants only a few studies have dealt with this question, and although some have reported a definite respiratory response to differences in latitude the evidence assembled so far does not indicate that the phenomenon is general in this category either.

Stocker (11) compared the respiratory rates of leaves from 2 species of Greenland shrubs with those of leaves from 3 species of trees in Java, and found them to be as high in the arctic plants at 10° C as they were in the tropical at 30° C. On the basis of this and other data assembled from the literature he concluded that many plants show a metabolic compensation for climatic conditions. The calculations were made on the basis of unit leaf area.

Wager (12) made respiratory studies at different temperatures on a series of arctic flowering plants from East Greenland, using twigs, shoots and leaves, and fresh weight as the unit of measurement. When these were compared with data on temperate zone plants, some of the northern plants showed indications of an increase in rate, but in many cases the figures for the 2 groups were not appreciably different.

A number of arctic and tropical lichens were compared with respect to respiratory rate between the temperatures 0° and 30° C by Scholander, Flagg, Walters, and Irving (10), and it was found that the values for the northern species largely coincided with the data obtained from the tropical species, although there was some indication of compensation in members of the families Peltigeraceae and Stictaceae.

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[2] Contribution no. 948 from the Woods Hole Oceanographic Institution. The work was carried out on the Blue Dolphin Labrador Expedition, 1954, aided by a contract between the Office of Naval Research, Department of the Navy, and the Arctic Institute of North America.

The plants which have been used for comparison often have come not only from different environments, but also from widely different species, and the question arises as to what extent such taxonomic or morphological differences may influence the results. Since the arctic flora includes many species, however, which extend into temperate regions through more or less distinct morphological taxa, an attempt has been made to minimize the taxonomic differences by limiting the present investigation to northern and southern material taken from the same species.

During an expedition to northern Labrador in the summer of 1954 respiratory measurements were made on 3 species of flowering plants, 1 of lycopodium, 2 of equisetum, and also on 2 lichens. Comparative records from material of the same species were obtained in Massachusetts and New Hampshire.

MATERIAL

The material is listed below with its range of occurrence (4, 5, 7): *Cladonia alpestris* s.l. (L.), from Central Baffin Is. to Florida; *Cladonia rangiferina* s.l. (L.), from Ellesmere Is. to Florida; *Cladonia sylvatica* s.l. (L.), from Central Baffin Is. to Florida; *Equisetum arvense* L., from N. Greenland to Virginia; *Equisetum sylvaticum* L., from N. Labrador to Pennsylvania; *Lycopodium annotinum* L., from W. central Greenland to Virginia mts.; *Arenaria peploides* L., from N. Greenland to Maryland; *Epilobium angustifolium* L., from Central Baffin Is. to N. Carolina mts.; and *Campanula rotundifolia* L., from N. Baffin Is. to W. Virginia.

The Labrador plants were collected on the arctic tundra north of the tree line in the vicinity of Hebron Fjord, at about latitude 58° 10′ N. during July and early August. Data on the arctic examples of *Cladonia sylvatica* were taken from the observations of

TABLE I

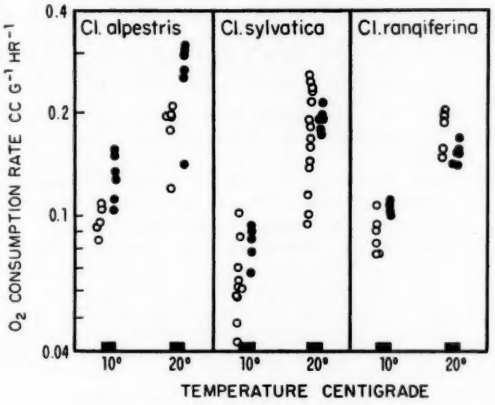
AVERAGE AIR TEMPERATURES IN °C FOR THE SUMMER MONTHS

	HEBRON, LABR. (6)	CAPE COD, MASS. (2)	HANOVER, N. H. (2)
May	0	12	12
June	4	17	18
July	10	20	19
Aug.	10	19	18
Sept.	4	16	14
Oct.	1	14	11

Scholander, Flagg, Walters, and Irving (10) on plants collected frozen at Point Barrow, Alaska, in October, and thawed in the laboratory.

Most of the comparative temperate zone material was collected around Woods Hole, Massachusetts, at about latitude 41° 30′ N. from the latter part of May to July, and the lichens in late October. The southern forms of *Campanula rotundifolia* and *Equisetum sylvaticum* were obtained in July near Hanover, New Hampshire, at about latitude 43° 43′ N.

Average air temperatures for the summer months at Hebron in Labrador, on Cape Cod, and at Hanover are given in table I. A comparison of microclimates would undoubtedly reveal much greater differences between the northern and southern localities, considering that the arctic plants were growing exposed on the tundra usually less than a foot above permafrost.



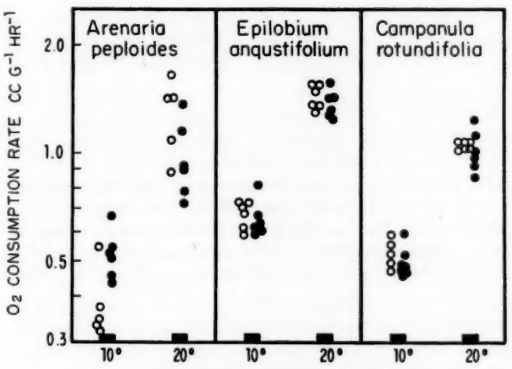


FIG. 1. Rates of O_2 consumption at 10° and 20° C in 3 species of Cladonia (above) and in 3 flowering plants (below). Units of O_2 consumption rate are; cc dry gas (N.T.P.)/g dry wt/hr. The arctic plants (open circles) are drawn to the left of the temperate zone plants (filled circles). Each point represents a single determination. The data on the arctic form of *Cladonia sylvatica* were taken from Scholander, Flagg, Walters, and Irving (10).

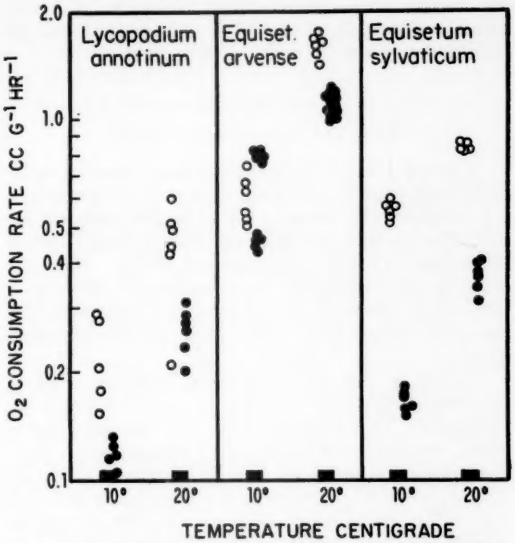


FIG. 2. Rates of O_2 consumption at 10° and 20° C in a lycopodium and 2 equisetums. Units of O_2 consumption rate are: cc dry gas (N.T.P.)/g dry wt/hr. The arctic plants (open circles) are drawn to the left of the temperate zone plants (filled circles). Each point represents a single determination.

EXPERIMENTAL

Respiratory measurements were made at 10° and 20° C on both arctic and temperate zone material, using a volumetric micro-respirometer (8). For each temperature at least 6 individuals of the plant selected for testing were picked generally in the vicinity of the laboratory. They were kept moist and prepared for the experiment as soon as possible. Care was taken that the northern and southern material used for comparison be as similar as possible in size and age. Fresh leaves of Campanula and Epilobium and branches of Equisetum were taken from the upper part of the stems. From Lycopodium and Arenaria branch tips 2 to 3 cm long were used. The bunches of Cladonia were separated and cleaned in water. The material was placed in 25 cc vials with a few drops of water, including whenever possible in each vial leaves from several different plants. The vials were covered with black masking tape, and the Lucite attachment buttons were blackened with ink. NaOH was used as CO_2 absorber. The temperature was kept constant to ± 0.1° C. After an equilibration period of about one half hour, readings were taken every 10 minutes, and the rates remained in general quite constant for the duration of

the experiments—approximately 2 hours. The material was then dried at 100° C and weighed, and all data are reported on a dry weight basis.

Results and Conclusions

Figures 1 and 2 are semilog graphs showing metabolic rates plotted against the centigrade temperatures.

Although the spread of data within each species often was as high as 100 %, differences from 1 species to another in some cases stand out clearly. Thus the rates of the 3 Cladonias were consistently lower than those of the other plants, and those of Lycopodium also were somewhat lower than those of the flowering plants.

It will be seen that 7 out of the 9 species of plants examined displayed no significant difference in respiratory rate between arctic and southern specimens, when measured at the same temperature. Only *Lycopodium annotinum* and *Equisetum sylvaticum* (fig 2) showed significantly higher rates at both 10° and 20° in the Arctic. This may reflect an adaptation to a colder climate, and it may also, of course, be due to differences in genetic material.

Therefore it appears that respiratory temperature compensation may be a factor of only minor importance for many arctic terrestrial plants, and that they must depend largely upon other conditions for survival in the cold, as do the insects in the Arctic.

Summary

The respiratory rates of leaves or shoots of 3 species of flowering plants, a lycopodium, 2 equisetums, and 2 lichens, growing in northern Labrador, have been compared with measurements from the same species of plants growing in the temperate zone. When taken at the same temperatures, the records for 7 out of 9 species showed no statistically significant difference between northern and southern specimens, and therefore no evidence of climatic compensation in respiratory rate. In the remaining 2 species (1 lycopodium and 1 equisetum) the rates were significantly higher for the northern plants than for their southern counterparts.

We are greatly indebted to Dr. P. F. Scholander and Dr. J. W. Kanwisher for help and guidance. We wish to thank Commander David C. Nutt of Dartmouth College, Master of the Blue Dolphin, for his interest and cooperation in Labrador and Hanover and Professor C. L. Wilson for providing us with working space at Dartmouth College. Mr. P. Savalov of the Institute of Zoophysiology, University of Oslo, has given us able assistance with the drawings.

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FACTORS IN CORN STEEP WATER PROMOTING GROWTH OF PLANT TISSUES[1, 2]

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Recently, we have been trying to maintain sterile cultures of tissues taken from plants which are especially responsive to the length of the daily photoperiod. We expect that in the process of establishing the cultures, particular requirements for growth substances by the various species will become evident; correlations between these requirements and sensitivities to the light and dark regimes will be sought. We also hope to learn whether or not the influence of inductive photoperiodic treatments given to intact plants can be maintained in cultures of tissues derived from such plants. If the influence is maintained, a good method for studying the nature of the influence will be available. In addition, proper control of the growing conditions may cause formation of small, sterile plants (for example, see (7)) which could be used in testing various preparations for the presence of substances such as the hypothetical flowering hormone.

The cocklebur (*Xanthium pennsylvanicum*) is widely known for its extreme sensitivity to the photoperiod; therefore, we have tried first of all to establish cultures of tissues taken from this plant. Our early efforts to obtain growth on various media containing indole-3-acetic acid and kinetin (6-furfurylamino-purine), coconut milk, malt extract, casein hydrolysate, and yeast extract were unsuccessful. Continuous growth finally was obtained when the excised tissues were placed on a medium containing corn steep water. A program to learn the nature of the growth-promoting material in the steep water is now underway. Some properties of this material and its interaction with other substances are discussed in this paper.

The callus tissue was derived from the cambial region of cocklebur stem segments. The plants used had been growing under long daily photoperiods in the greenhouse for about 10 weeks and had been started from seeds kindly sent to us by Dr. James Bonner of the California Institute of Technology. Leaves were trimmed from long sections of stem, and the bark was removed so as to keep the inner tissues in a sterile condition. The remaining cylinder was then cut into pieces about 1 cm long which were placed in Petri dishes. Tangential slabs were cut from these pieces and planted on the surface of an agar medium in 125 ml Erlenmeyer flasks. The flasks were kept in a room at 27° C continuously but dimly lighted (about 25 ft-c) by warm white fluorescent lamps. The medium contained (mg/l): $Ca(NO_3)_2$, 100; KNO_3, 80; $MgSO_4$, 35; KCl, 65; KH_2PO_4, 12; NH_4NO_3, 400; KI, 0.8; $MnSO_4$, 4.4; $ZnSO_4$, 1.5; H_3BO_3, 1.6; sodium ferric ethylenediaminetetraacetate, 32; glycine, 2; thiamine · HCl, 0.1; nicotinic acid, 0.5; pyridoxine · HCl, 0.1; indole-3-acetic acid, 2; sucrose, 20,000; and agar, 10,000. This and all other media were adjusted to pH 6.0 before autoclaving for 15 minutes. On this medium (referred to as basal medium), a white callus developed in the cambial region at the basal end of each slab. Such callus soon stopped growth and did not develop further when cut up and transferred to fresh supplies of the substrate. Growth did continue, however, when some kinetin and corn steep water were added to the medium just described. By now, the tissues have been sub-cultured at least 16 times, transfers usually being spaced 3 to 5 weeks apart. Large amounts of tissue have been generated from the few beginning pieces.

Corn steep water, a readily available by-product of the corn wet-milling industry, is manufactured by allowing shelled corn to soak in open wooden tanks at 45 to 52° C for 40 to 48 hours. Five to seven gallons of water are used for every bushel of corn. Lactic acid bacteria are abundant in the corn steep water and maintain the pH at the level considered most favorable for milling operations (8). Steep water with the highest concentration of dissolved solids is withdrawn from the system and evaporated to a solid concentration of approximately 55%. This concentrate has been used extensively as a nutrient medium for microorganisms and has been found to stimulate the biosynthesis of penicillin (6). For an extensive summary of the composition and properties of corn steep water as well as its manufacture and use in culture media, see Liggett and Koffler (3).

The corn steep water, as generously supplied by Dr. Stanley Watson of the Corn Products Refining Corporation, contained 669 mg of solids per ml. As an initial purification step, the steep water was adjusted to 80% ethanol by addition of 95% ethanol. The material active in stimulating callus tissue growth stayed in solution but a copious precipitate of inactive material formed. After the alcohol was distilled from the supernatant, the remaining water solution was layered 3 times with single volumes of diethyl ether. The activity remained in the dark brown water layer which then contained 267 mg dry material per each starting ml of steep water. This cleaned-up water solution was designated preparation F and has been

[1] Received February 24, 1959.

[2] This work was supported by a grant from the Graduate School of Indiana University to C.M.

TABLE I

CORRELATION BETWEEN THE CONCENTRATION OF CORN STEEP PREPARATION F AND GROWTH OF COCKLEBUR CALLUS*

CONCENTRATION PREPARATION F	FINAL FRESH WEIGHT/PIECE	FINAL DRY WEIGHT/PIECE
ml/100 *ml medium* **	*mg*	*mg*
0	48	7
0.1	138	16
0.3	209	22
0.5	243	24
0.7	357	36
1.4	385	36
2.0	349	32

* Twelve pieces of tissue for each concentration. Thirty-one days of growth. Initial fresh weight/piece, 24 mg; initial dry weight/piece, 2 mg.

** All media also included 2×10^{-5} M kinetin.

used in maintaining stock cultures and as a reference of activity for all subsequent fractionations. It has been added at the optimal concentration (table I) equivalent to 0.7 ml of the original corn steep per each 100 ml of culture medium.

The active material was destroyed by ashing, but apparently was not affected by boiling for 5 minutes at pH 1.2, 6.0, or 11.5. Although soluble in water and 80 % ethanol the material did not go into diethyl ether at pH 2.5, 6.0, or 11.0 nor into *n*-butanol at pH 3.0. The active fraction passed readily through a collodion dialysis membrane. Attempts to precipitate the material with barium (pH 8.2 with 3 volumes of ethanol added) and with silver (pH 3.0 and 6.9) were unsuccessful; in no case, however, was full activity detected in the supernatants.

Part of the activity was adsorbed onto and eluted from Merck activated charcoal. In 1 experiment which gave very striking results, 12 ml of preparation F were added to the top of a column of the charcoal and forced through by compressed air at a pressure of about 15 lbs/sq in. The column had a diameter of 1.2 cm and was 16 cm long. The F preparation was followed by 80 ml of distilled water and the 2 colorless effluents were pooled. Next, 80 ml of 1 *N* NH_4OH were added to the column and the effluent, again colorless, was collected. This was followed by the addition of 80 ml of 100 % pyridine and a 3rd, dark brown effluent was obtained. From these fractions the ammonia was removed by boiling and the pyridine by extracting into diethyl ether after being mixed with water. In other runs of essentially identical setups, a 3 : 1 mixture of 95 % ethanol and concentrated NH_4OH was used instead of the pyridine and gave similar results in terms of growth effects. The 1st effluent including the water wash always caused an increased growth of the tissues (table II) but much less than obtained with the F preparation. The resulting callus was firm and green. The 1 *N* NH_4OH eluate did not influence growth to any extent. When grown on either the pyridine or ammonia-alcohol eluate the tissues usually become brown and watery in appearance. Addition to the basal medium of a combination of the water effluent and the pyridine eluate, however, always led to healthy, vigorously growing tissues and to greater increases of tissue weights than were obtained on either alone. Furthermore, the total growth increase on the combination was always greater than the sum of the growth increments caused by either alone (table II). Therefore, it is very likely that at least 2 factors in the corn steep are necessary for really excellent growth of the cocklebur tissues. Supporting this idea is the fact that increasing the concentration of either fraction does not cause more growth than that occurring at the usual test level. Also, when the water effluent is run through a 2nd charcoal column, no activity seems to be adsorbed to the charcoal. The extent of purification achieved is indicated roughly by the fact that the water effluent contains 102 mg and the ammonia-alcohol eluate 48 mg dry material for each starting ml of corn steep.

The very striking increase of growth on the corn steep medium occurred only if a large amount of kinetin was also present (Expt A, table III). The optimal concentration of this substance was 2 to 3×10^{-5} M or slightly higher in a medium containing 7 ml of preparation F per liter (Expt B, table III). Kinetin caused some increased growth even in the absence of added corn steep although the tissues were not so uniform and vigorous. It may be that this effect of kinetin alone was dependent upon a carry-over of corn steep factors from the previous substrate. The high concentration of kinetin necessary is in sharp contrast to the low levels required in culture of some other tissues such as tobacco (5) in which excellent growth occurs with only 10^{-6} M and in which 2×10^{-5} M may be inhibitory. It will be interesting to learn whether or not such quantitative differences in kinetin requirements are correlated with sensitivities to the light and dark periods.

Attempts to replace the kinetin with adenine were not successful. However, at a high concentration of adenine (3.7×10^{-3} M) some callus growth occurred and roots appeared. Essentially this same type

TABLE II

ENHANCEMENT OF COCKLEBUR CALLUS GROWTH BY THE WATER EFFLUENT AND PYRIDINE ELUATE FROM THE CHARCOAL COLUMN *

MEDIUM**	FINAL FRESH WEIGHT/PIECE	FINAL DRY WEIGHT/PIECE
	mg	*mg*
Basal	71	7
Basal + water effluent	120	13
Basal + pyridine eluate	78	7
Basal + water effluent + pyridine eluate	285	25

* Twelve pieces of tissue per test. Thirty-one days of growth.

** All media also contained 2×10^{-5} M kinetin. Effluent and eluate each were used at a concentration equivalent to 1 ml corn steep/100 ml medium.

TABLE III

EFFECT OF KINETIN ON GROWTH OF COCKLEBUR CALLUS*

ADDITIVE TO BASAL MEDIUM	FINAL FRESH WEIGHT/PIECE	FINAL DRY WEIGHT/PIECE
	mg	*mg*
Expt A		
None	16	2
Preparation F (7 ml/l)	89	12
Kinetin (2×10^{-5} M)	183	19
Preparation F (7 ml/l) + kinetin (2×10^{-5} M)	511	48
Expt B **		
0	48	7
0.1×10^{-5} M kinetin	73	9
1×10^{-5} M "	151	15
2×10^{-5} M "	192	19
3×10^{-5} M "	209	20
5×10^{-5} M "	210	18

* Twelve pieces of tissue per test. Thirty-one days of growth.

** Each liter of medium also contained 7 ml preparation F.

of growth occurred on 10^{-6} M kinetin. Here, then, is another example (some others in (2, 7)) of the similarity of kinetin and adenine effects on growth with the kinetin being effective at much lower concentrations and having a much more striking influence. The possibility that kinetin serves as a highly efficient adenine is still attractive.

Cocklebur tissues planted on a corn steep medium containing no added indoleacetic acid grew well. They grew better, however, on similar media containing 2.8-22 $\times$ 10^{-6} M added auxin. The growth occurring on the medium without the added indoleacetic acid may have been dependent upon auxin carried over from the previous medium or, more likely, upon auxins and auxin-precursors which are known to abound in corn endosperm (1). The acid in free form presumably would have been removed in the ether extractions but other forms and precursors may have remained in the water layer.

Promotion of growth by corn steep water is not confined to cocklebur tissues. Callus derived from the cambial region of stems of *Helianthus annuus* responds very strikingly to the steep and, furthermore, does much better on a combination of both the water effluent and the ammonia-alcohol eluate from the charcoal column than on either fraction alone. Therefore, the response of the sunflower is much like that of the cocklebur. The growth of tissues from carrot, balsam, bush bean, and lettuce also definitely are promoted by the steep water. The possibility for a much wider use of corn steep in culture media seems bright.

Our studies are not sufficiently complete to permit a critical comparison between the active corn steep factors and the substances in corn endosperm which have been used to culture immature embryos (4). Once we have achieved identification of the factors, we will be able to make a direct comparison and to study more clearly the role of the substances in plant growth.

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USE OF SPECIFICALLY LABELED GLUCOSE AND GLUCONATE IN THE EVALUATION OF CATABOLIC PATHWAYS FOR GLUCOSE IN CORN ROOTS [1, 2, 3]

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In a previous paper from this laboratory (7) it was reported that root tips from 2,4-dichlorophenoxyacetic acid (2,4-D)-treated pea, corn or oat seedlings converted the 1st carbon of glucose to CO_2 more rapidly than did the controls, but CO_2 production from the 6th carbon was not changed appreciably. It was concluded from these results that 2,4-D increased the amount of glucose catabolised through the pentose phosphate pathway. If this conclusion is correct, then a larger proportion of the glucose catabolised by the 2,4-D-treated tissues should follow the same pathway as gluconate. This greater similarity of pathway should show up when $C^{14}O_2$ production rates from specifically labeled glucose and gluconate are measured and compared. The results of such experiments are presented in this report. The point is made that it is not possible to quantitatively evaluate catabolic mechanisms for glucose from only a comparison of the yields of $C^{14}O_2$ from the 1st and 6th carbons of glucose.

MATERIALS AND METHODS

Corn seedlings (*Zea mays* L., var. Funks G-50) approximately 60-hours-old were used as plant material. The seedlings were placed with their roots in 10^{-2} M potassium phosphate buffer, pH 5.3, which contained 2,4-D or indoleacetic acid (IAA) at the concentrations given in the text. Control seedlings were treated with buffer alone. During the 12-hour treatment period the seedlings were placed in the dark at 22 to 23° C. After treatment, root tips (2 cm long) were cut from the seedlings, weighed in groups of 7 (about 180 mg fresh weight) and placed in 15 ml Warburg vessels. In addition to the root tips, each vessel contained 2.1 ml potassium phosphate buffer, pH 5.3, 0.1 ml C^{14} substrate (25 micromoles; approximately 45,000 cpm in the case of gluconate, approximately 75,000 cpm in the case of glucose), 0.2 ml of 20 % carbonate-free NaOH (center well) and 0.2 ml of 10 *N* H_2SO_4 (side arm). The vessels were shaken in the dark for 3.5 hours at 25° C, and the $C^{14}O_2$ released in respiration was collected in the NaOH of the center well. Detailed experimental procedure is given in a previous paper (7).

Glucose-1-C^{14} (G-1-C^{14}) and glucose-6-C^{14} (G-6-C^{14}) were purchased from Volk Radiochemical Co. or from Nuclear-Chicago Corp. Glucose-2-C^{14} (G-2-C^{14}) was purchased from National Bureau of Standards. The sodium salts of gluconate-1-C^{14}, gluconate-2-C^{14} and gluconate-6-C^{14} were prepared by hypoiodite oxidation (9) of the corresponding labeled glucose. 2,4-D (Eastman Kodak Co.) was neutralized with NaOH and the sodium salt was recrystallized twice from a water-alcohol solution. IAA (reagent grade) was purchased from Fisher Scientific Co., and used without further purification.

RESULTS AND DISCUSSION

The percent of the added C^{14} (glucose-C^{14} or gluconate-C^{14}) recovered as $C^{14}O_2$ was determined for

TABLE I

EFFECT OF 2,4-D AND IAA ON $C^{14}O_2$ PRODUCTION FROM SPECIFICALLY LABELED GLUCOSE AND GLUCONATE BY CORN ROOT TIPS

EXPT	TREATMENT *	POSITION LABELED	% C^{14} ADDED RECOVERED AS $C^{14}O_2$	
			GLUCOSE	GLUCONATE
1	Control	1	0.93	1.40
		2	0.50	0.21
		6	1.05	0.13
2	"	1	1.21	1.50
		2	0.65	0.36
		6	1.18	0.19
3	10^{-3} M 2,4-D	1	1.95	1.45
		2	1.45	0.85
		6	1.10	0.60
4	"	1	2.05	1.79
		2	1.65	1.15
		6	1.03	0.62
5	10^{-3} M IAA	1	1.34	0.88
		2	0.72	0.37
		6	0.79	0.26
6	"	1	1.25	0.86
		2	0.72	0.44
		6	0.82	0.30
7	10^{-5} M 2,4-D	1	1.66	1.59
		2	0.75	0.55
		6	1.20	0.35
8	10^{-5} M IAA	1	1.35	1.78
		2	0.74	0.49
		6	1.15	0 31

* For treatment procedure see text.

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each substrate and compared for each treatment. However, since 2,4-D inhibits by about 50 % the uptake of glucose by corn roots (8), a more meaningful comparison between treatments can be made by comparing ratios derived from $C^{14}O_2$ yield data of specifically labeled carbon atoms within a single compound. This method was adopted for the experiments reported here. $C^{14}O_2$ yield data and the derived ratios are given in tables I and II, respectively.

The C_6/C_1, C_1/C_2 and C_6/C_2 ratios obtained when root tips from the control seedlings were used (expts 1 and 2) indicate that glucose and gluconate do not share, for the most part, the same pathways of metabolism. Furthermore, that the values for the C_6/C_1 (glucose) ratios for the control root tips approximated 1.0 suggests the glycolytic pathway as the major one in glucose metabolism. On the other hand, the high values obtained for the C_6/C_2 (glucose) ratios argue against this conclusion. Considering the 2nd and 6th carbons of glucose as giving rise (after glycolytic degradation) to the carbonyl and methyl carbons of acetyl coenzyme A, it would be expected that the C_6/C_2 ratio would be well below 1.0, not above 1.0 as was found. Experiments using acetate-1-C^{14} and acetate-2-C^{14} have given results of 0.26 to 0.34 for the C_2/C_1 (acetate) ratio (8).

The high values obtained for the C_6/C_2 (glucose) ratio are surprising, but they are believed to be correct. The radioactivity of the added glucose was checked repeatedly, and it is not likely that the results were due to a systematic error in the estimation of the C^{14} content. It should be noted that other workers also have reported high values for the C_6/C_2 (glucose) ratio. Agranoff et al (2) obtained a ratio of 0.5 using rat liver slices. When liver slices from rats fasted 48 hours were used, however, the ratio increased to 1.6. The increase in the ratio after fasting was due to a decrease in the yield of $C^{14}O_2$ from G-2-C^{14}. Barbour et al (3) obtained ratio values above 1.0 during the first 4 hours after the addition of C^{14} glucose to tomato fruits. Both groups of investigators reported, however, a considerably higher yield of $C^{14}O_2$ from G-1-C^{14} than from G-6-C^{14}.

The recent work of Rose (10) provides a possible explanation of the high C_6/C_2 (glucose) ratio. In estimating the degree of participation of pathways of glucose catabolism it is assumed that fructose-1,6-diphosphate, dihydroxyacetone phosphate and glyceraldehyde phosphate are in isotopic equilibrium. The results of Rose (10) indicate, however, that this assumption might not be justified. Rose found that crystalline muscle aldolase catalyzed an exchange reaction between the 4, 5, 6 carbons of fructose-1,6-diphosphate and glyceraldehyde phosphate. Such an exchange could result in a greater yield of $C^{14}O_2$ from G-6-C^{14} than from G-2-C^{14}. It is apparent from these results that quantitative evaluation of catabolic mechanisms for glucose based only on C_6/C_1 (glucose) ratios is not possible.

Beevers and Gibbs (5) and Beevers (4) have ascertained the position of C^{14} in ethyl alcohol formed from labeled glucose, gluconate and pentose by corn root tips and other tissues. From the position of the label in alcohol they concluded that glucose underwent glycolytic degradation, while gluconate and the pentose were degraded via the pentose phosphate pathway. Their results constitute good evidence for the occurrence in vivo of glycolytic and pentose phosphate pathways which are the same as those formulated from in vitro studies. However, since either anaerobiosis or the use of arsenite was necessary to obtain the required alcohol production, it is not possible to evaluate the degree of participation of the pathways in "normal" corn roots from their results.

In contrast to the results obtained with the controls, the results when root tips from 10^{-3} M 2,4-D treated seedlings were used yielded ratio values which were much the same for glucose as for gluconate (expts 3 and 4), suggesting that in these tissues glucose and gluconate were metabolized for the most part via the same pathway; presumably the pentose phosphate pathway. From a consideration of the C_6/C_1 (glucose) ratio alone, it would be concluded that a minimum of one half of the glucose was catabolized via the pentose phosphate pathway (6). However, comparing the glucose and gluconate ratios (expts

TABLE II

RATIO VALUES DERIVED FROM THE PERCENT RECOVERIES OF $C^{14}O_2$ WHEN CORN ROOTS WERE SUPPLIED WITH C^{14} GLUCOSE OR C^{14} GLUCONATE

EXPT	TREATMENT *	GLUCOSE			GLUCONATE		
		C_6/C_1**	C_1/C_2	C_6/C_2	C_6/C_1	C_1/C_2	C_6/C_2
1	Control	1.13	1.86	2.10	0.10	6.67	0.62
2	"	0.98	1.86	1.82	0.13	4.20	0.53
3	10^{-3} M 2,4-D	0.56	1.34	0.76	0.40	1.71	0.71
4	"	0.50	1.24	0.62	0.35	1.56	0.54
5	10^{-3} M IAA	0.66	1.74	1.14	0.35	1.94	0.68
6	"	0.59	1.86	1.10	0.30	2.40	0.70
7	10^{-5} M 2,4-D	0.72	2.21	1.60	0.22	3.00	0.64
8	10^{-5} M IAA	0.85	1.82	1.55	0.17	3.65	0.63

* For treatment procedure see text.

** Percent of C^{14} added as G-6-C^{14} appearing as $C^{14}O_2$ / Percent of C^{14} added as G-1-C^{14} appearing as $C^{14}O_2$

3 and 4), it seems quite likely that the pentose phosphate pathway accommodated considerably more than one half the total glucose catabolized.

The ratio values obtained after treatment with 10^{-3} M IAA (expts 5 and 6) were intermediate between those obtained with control seedlings and those obtained with the 10^{-3} M 2,4-D-treated seedlings, suggesting that IAA caused some increase in the participation of the pentose phosphate pathway. Treatment with more dilute concentrations (10^{-5} M) of 2,4-D or IAA (expts 7 and 8) also resulted in ratio values for glucose that were closer to those obtained with gluconate than was the case with the control root tips.

Comparisons of the values of ratios derived from $C^{14}O_2$ yields from specifically labeled glucose and gluconate permit a semi-quantitative conclusion as to pathway participation only if the ratio values for the 2 compounds are similar. Dissimilarity such as was found using the control root tips permits only a qualitative conclusion as do the results with 10^{-3} M or 10^{-5} M IAA and 10^{-5} M 2,4-D.

The rates of $C^{14}O_2$ production from the 2nd and 6th carbons of gluconate were increased relative to that of the 1st carbon as a result of treatment with the auxins. This was reflected in an increase in the C_6/C_1 ratio and a decrease in the C_1/C_2 ratio. However, the C_6/C_2 (gluconate) ratios were remarkably constant regardless of treatment. Apparently the auxins increased the rate of pentose degradation relative to the rate of pentose formation from gluconate, but did not affect the relative rates of $C^{14}O_2$ production from C-1 and C-5 of the pentose.

The results obtained using 10^{-5} M 2,4-D and IAA are of particular interest because treatment at this concentration elicited a growth response in the roots which resulted in the development of a swollen area starting 3 to 4 mm from the root tip and extending 4 to 5 mm along the root axis (for a discussion of this growth phenomenon see ref. (1)). Although the inferred increase in the participation of the pentose phosphate pathway due to 10^{-5} M 2,4-D or IAA was small, it nevertheless may be directly related to the growth response. This eventuality warrants continued investigation.

Summary

The yields of $C^{14}O_2$ from glucose and gluconate labeled in the C-1, C-2 or C-6 positions were used to evaluate the extent of the participation of the pentose phosphate pathway during glucose catabolism in root tips of corn seedlings. It is concluded that the pentose phosphate pathway did not participate in glucose catabolism to any great extent.

The yield of $C^{14}O_2$ from the 2nd carbon of glucose was only about one half the yield from the 1st or 6th carbon of glucose. These results are discussed and a possible explanation presented. The point is made that a quantitative evlauation of catabolic mechanisms for glucose based only on the $C^{14}O_2$ yields from the 1st and 6th carbons of glucose is not possible.

The effect of pre-treatment of the seedlings with 2,4-D or IAA on the participation of the pentose phosphate pathway also was investigated. It is concluded that 2,4-D and IAA caused an increase in the participation of this pathway; when 10^{-3} M 2,4-D was used, glucose catabolism was almost totally accommodated by this pathway.

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AN INHIBITOR OF OXALIC ACID OXIDASE IN BEET EXTRACTS[1]

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Oxalic acid oxidase, studied in spinach leaves by Laties (7) and in sugar beet leaves by both Arnon and Whatley (1) and Finkle and Arnon (5), has been demonstrated by the present authors in a thin peripheral layer of beet roots. The enzyme is here associated with a particulate constituent of the cells, probably mitochondrial in nature. Extreme fluctuations in activity were encountered, so that active preparations usually could be obtained with ease from the small (red) roots of commercial "beet greens" and also from young sugar beet roots grown in the greenhouse, but not from more mature sugar beet roots harvested in the greenhouse in July and August. Laties (7) has reported a phenomenon for spinach which is at least remotely similar: Leaves of plants grown at 26° C yielded active preparations, but those of specimens raised at 18° C did not. He did not analyze this situation.

In Beta, the present authors found that homogenates of older sugar beet roots strongly inhibited the oxalic oxidase activity of red beet particles. This fact argues in favor of the possibility that, in the older beet roots themselves, the oxalic acid oxidase is not lacking but is merely suppressed in its activity by some inhibitor. The present article demonstrates that this is indeed the situation. Identification of the inhibitor was made possible by substituting a solution of moss oxalic acid oxidase for the particulate enzyme from red beet. In addition to being a more convenient method, this allowed a more quantitative approach.

MATERIALS AND METHODS

The beets used belonged to the species *Beta vulgaris* Linn. Most experiments were performed with sugar beets, which are sometimes designated as var. saccharifera; however, there does not seem to be a real difference between this variety and the var. crassa comprising most of the cultivated beets.

Moss oxalic acid oxidase was extracted from *Mnium affine* Bland. Six g of Mnium powder was extracted overnight at 4° C in a ball mill with 250 ml 6 % NaCl. After pouring off the thick slurry obtained, the ball mill and its pebbles were rinsed with 50 ml of water and the fluids were combined. Solids were eliminated by centrifugation at 30,000 × G for 20 minutes and the ice-cold, water-clear supernatant was adjusted to pH 4.5. Solid ammonium sulfate was added to give 75 % saturation. The precipitate was spun down at 30,000 × G for 20 minutes, taken up in 25 ml of ice-cold water and dialyzed against cold distilled water for 6 hours with constant stirring and several changes of water. A slight precipitate was eliminated by centrifugation. The clear supernatant represented the "moss enzyme" referred to in the text.

Beet extracts containing the inhibitor were prepared originally by homogenizing the beets in a Waring blendor with ice-cold water. In later experiments, this was always followed by high speed centrifugation, since the inhibitor turned out to be present in the clear supernatant. The effect of the beet inhibitor on the catalytic activity of the moss oxidase extract was measured manometrically at 21° C using Warburg vessels containing mixtures of the following composition: Main compartment, 0.5 ml moss enzyme extract, 1.0 ml inhibitor (omitted in the control vessel), 0.5 ml 0.05 M phosphate buffer (pH 4.6), 0.2 ml 10^{-5} M riboflavin phosphate, 0.2 ml 2 % sodium-ethylenediaminetetracetic acid (Na-EDTA); side arm, 0.2 ml 0.1 M oxalic acid. The chelating agent, EDTA, was added to the reaction mixture because of Datta's finding (2) that certain cations normally present in moss extracts have a deleterious effect on oxalic acid oxidase activity. The riboflavin phosphate was added because of the observation by Datta (2) and Datta and Meeuse (3) that this substance stimulates the activity of the oxidase under the conditions of this test. After a 10 minute equilibration period the Warburg manometer stopcock was closed and contents of side arm and main compartment were mixed. The R.Q. for oxalic acid oxidation was 4 with or without the inhibitor present. CO_2 produced in excess of O_2 consumed was measured in duplicate vessels, usually over a 20 minute period during which approximately 400 μl gas evolution occurred. The reaction proceeded at a rate which was almost constant.

Recognizing that the experimental mixture was not very effectively buffered at pH 4.6, some experiments were run with ascorbate buffer. However this was not regarded as sufficiently inert biochemically and its use was not continued. Since the reaction proceeded almost linearly with time, and the rate data were adequately reproducible, the experimental mixture was considered satisfactory for present purposes.

Ether extractions of beet juices were carried out with the aid of a Kutscher-Steudel apparatus as described by Umbreit et al (8).

Nitrate was determined by the method of the American Potash Institute (4).

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Results

In table I, experiment 1 shows that the beet inhibitor is present in solution. Experiment 2 demonstrates that it is thermostable and active even in high dilution. From experiment 3 it is clear that the inhibitor must be a substance of low molecular weight moving readily through a dialysis membrane. Experiment 4 indicates that it is not an artifact such as a quinone arising during the preparation of the beet extract through tyrosinase activity. The reversibility of the inhibitory effect (experiment 5) suggests that some organic acid naturally present in beets and acting as a competitor of oxalic acid is responsible, but this is contradicted by the observation that the inhibitor is not ether-extractable (experiment 6). This leaves (among other possibilities) the chance that the inhibitor is some simple salt. To check this point, a comparison was now made between richly-fertilized beet plants with dark-green foliage and poorly-fertilized ones, somewhat paler in appearance. When mixed with water so as to achieve 90 % inhibition in the standard test system, the press juice of whole plants had to be diluted 150 times in the case of the well-fertilized plants and only 9 times for the others, a result which certainly favors the salt-hypothesis. Further information was thereupon obtained with the aid of ion exchange resins. It was found that removal of the cations with Amberlite IR-120 had no effect on the inhibitory properties of beet juice, whereas elimination of the anions with Amberlite IRA-400 abolished the inhibition completely. Since possible pH-effects in these experiments were avoided or counteracted, it was concluded that the inhibitor is anionic in nature.

At this point, the authors felt that data on the distribution of the inhibitor in the beet plant might provide further clues. Leaves, petioles and roots were analyzed separately, and a striking pattern emerged; the concentration of inhibitor turned out to be low in the leaves, extremely high in the petioles, and intermediate in the roots. Thus, leaves harvested in the afternoon usually had to be extracted with only twice their fresh weight of water to yield solutions causing partial inhibition (76 to 93 %); root extracts could be made 10 times more dilute to give the same effect, and petiole extracts 100 times more dilute.

Finally, it was observed that the inhibitor had a tendency to disappear from sugar beet leaves upon illumination. Comparable leaf samples, including the petioles, were selected from 1 well-fertilized greenhouse plant; 1 sample was analyzed immediately for inhibitor, the other after a continuous 70-hour exposure to light. The illuminated leaves (including petioles) were kept in an Erlenmeyer flask with distilled water which was changed frequently to preclude the development of microorganisms. Only leaves maintaining a completely healthy appearance were used. Whereas, before illumination, the leaf-extract inhibited moss oxalic acid oxidase so severely that only 3 % of the activity was left, there was only partial inhibition (85 %) after the illumination.

As a result of all the experiments recorded above, the problem was reduced to the following question: What anion, present naturally in well-fertilized sugar beets, possesses the distribution-pattern mentioned, and disappears from the leaves upon illumination? Nitrate seemed to fulfill the requirements. An inhibitory effect of nitrate on moss oxalic oxidase was reported by Franke et al (6) in 1937. The family of the Chenopodiaceae, to which spinach and beet belong, is noted for the tendency of some of its members to

Table I

Inhibition of Soluble Moss Oxalic Acid Oxidase by Beet Extracts after Various Treatments

Experiment Number	Treatment	Remaining activity	Inhibition %
	Control (no inhibitor added)	435 mm²	
	Control + inhibitor (beet homogenate)	20	95.5
1	Control + supernatant of inhibitor)	17.5	96
	Control + well-washed particles from inhibitor	287	34
	Control + inhibitor (beet extract)	13	97
2	Control + boiled inhibitor	11	97.5
	Control + boiled inhibitor diluted 1:10	15	96.5
	Control + inhibitor (beet extract)	13	97
3	Control + dialyzed inhibitor	339.5	22
	Control + concentrated dialyzate	17.5	96
	Control + boiled extract of fresh beet cubes	56.5	87
4	Control + extract of pre-boiled beet cubes	39	91
	Control + diffusate obtained in pre-boiling of beet cubes	43.5	90
	Control + inhibitor (beet extract)	15	96.5
5	Control mixed with inhibitor, then dialyzed (dil. 10/7)	304.5	30
	Control alone, dialyzed (dil. 10/7)	322	26
	Control + concentrated dialyzate	30.5	93
	Control + inhibitor (beet extract)	43.5	90
6	Control + ether-extracted inhibitor	35	92
	Control + redissolved residue of ether-extract of inhibitor	452.5	−4

accumulate nitrate. The reduction of this compound concomitant with photosynthesis is, likewise, well-known. In the case of the leaves just referred to, the following nitrate-values were found; before illumination, 1.496×10^{-4} M; after illumination, 0.464×10^{-4} M. It can still be argued that this result was due to some other factor and that there was no causal relationship between the drop in nitrate and the drop in inhibitory properties upon illumination; or, conceivably, the inhibition could have been caused by the combination of a multitude of factors. However, this can be tested easily. Should nitrate be the sole inhibitor, then it should be possible to account quantitatively for the effect exerted by beet extracts, on the basis of their nitrate content. Table II and figure 1, summarize the results of experiments with beet juice, as well as of those with pure nitrate and chloride solutions, and provide strong evidence confirming that nitrate alone can account for the inhibitory effects observed.

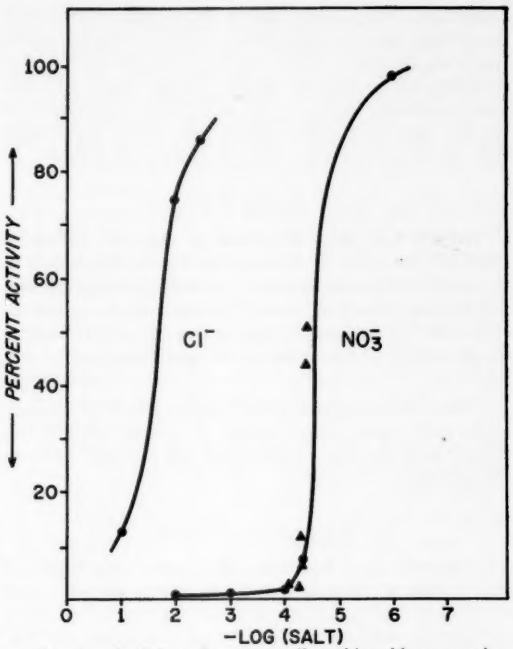

FIG. 1. Activity of moss oxalic acid oxidase remaining when the enzyme is acted upon by salt solutions and by beet extracts. Circles: experiments with pure salt solutions. Triangles: experiments with beet extracts (each point indicates the nitrate content of a given beet extract).

DISCUSSION

As yet, the importance of the work reported here is mainly practical. The fact that nitrate ions are able to paralyze the activity of oxalic acid oxidase in the remarkably low concentration of 5×10^{-5} M makes it imperative to avoid or eliminate these ions in studies of this particular enzyme, and to investigate very carefully their possible role in other cases. Nitrate also may become a biochemical tool for the suppression of oxalic acid oxidase; conversely, the latter enzyme might, in special situations, be used as a tool for the quick estimation of nitrate. However, the work here reported has theoretical implications also; e.g., the inhibition by nitrate may shed light on the mechanism of action of oxalic acid oxidase. Furthermore (although generalizations based on in vitro experiments, with complete neglect of the architecture of the cell, can at best be extremely speculative) it is tempting to contemplate a connection between photosynthesis and respiration (or at least oxalic oxidase activity) via nitrate and nitrate reduction. The present authors do not wish to make the claim that nitrate is in all cases responsible for the lack of oxalic acid oxidase activity observed in beet or spinach

TABLE II

REMAINING ACTIVITY OF OXALIC OXIDASE IN THE PRESENCE OF NITRATE AND CHLORIDE SOLUTIONS AND OF BEET EXTRACTS

AGENT CAUSING THE INHIBITION	REMAINING ACTIVITY	INHIBITION %
Control	405	...
Nitrate 10^{-6} M	393	3
Nitrate 10^{-5} M	344	15
Nitrate 5×10^{-5} M	28.5	93
Nitrate 10^{-4} M	8	98
Nitrate 10^{-3} M	4	99
Nitrate 10^{-2} M	4	99
Chloride 2×10^{-3} M	348.5	14
Chloride 10^{-2} M	300	26
Chloride 10^{-1} M	52.5	87
Beet petiole extract (5.73×10^{-5} M nitrate)	8	98
Beet leaf extract (5.0×10^{-5} M nitrate)	28.5	93
Extract I of beet leaf + petiole (1.11×10^{-4} M nitrate)	12	97
Extract II of beet leaf + petiole (5.20×10^{-5} M nitrate)	48.5	88
Extract of beet root core (3.63×10^{-5} M nitrate)	208.5	48.5
Extract of beet root skin (3.88×10^{-5} M nitrate)	180	55.5

particles. Thus, although in their own experiments extensive washing and dialysis of the oxidase-bearing particles generally led to active preparations, this was not the case in some of Finkle's experiments (Finkle, oral communication). The possibility must therefore be left open that other factors are (also) responsible for the "seasonal" variation in activity observed.

Summary

Nitrate has been identified as the sole factor in certain beet extracts responsible for the inhibition of oxalic acid oxidase in vitro. Under the experimental conditions described nitrate concentrations as low as 5×10^{-5} M sufficed to give severe inhibition. Some implications of the work have been discussed.

The work reported here forms part of an investigation of oxalic acid oxidase in mosses and higher plants supported by a grant from the National Science Foundation.

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News and Notes

American Society of Plant Physiologists, *Northeastern Section*: The 23rd annual meeting of the Section was held in Skinner Hall, University of Massachusetts, Amherst, Mass., on May 1 and 2, 1959. Because of the resignation of Chairman Theodore T. Kozlowski, Vice-chairman Conrad S. Yocum presided; he was ably assisted by Dr. Arthur C. Gentile, chairman of the local committee.

An audience of 97 members heard 29 research reports and, after Friday's dinner, was most entertainingly informed about his recent European trip by Professor F. C. Steward of Cornell.

The Northeastern Section had been petitioned to accept as full members those members of the parent society residing in Delaware and eastern Pennsylvania; an amendment permitting this action was passed without dissenting vote. Officers elected were: chairman, C. S. Yocum; vice-chairman, Stuart Dunn; secretary-treasurer, H. J. Dyer; executive committeeman, B. M. Pollock. Resignations from the Executive Committee were regretfully accepted from Professors Irene H. Stuckey and Charles J. Lyon, who were unanimously voted the Section's deep appreciation for their many years of devoted service.—Hubert J. Dyer, *Secretary-Treasurer*.

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2. The Manuscript Will be Sent out for Review by two competent reviewers familiar with the field of research covered by the paper. These reviewers may or may not be members of the Editorial Board. In case of marked disagreement between reviewers, the Editor will appoint a referee, usually a member of the Board, to make a recommendation for the Board. Usually two months suffices for review and decision of the Editorial Board.

3. Manuscripts Returned to the Author for Revision should be sent back to the Editor within two months or the paper will be published under the date of receipt of the revised manuscript and not the original date.

4. Galley Proof will be sent to the designated author (See below; Part II, Sec. 4) by the printer, but it should be returned directly to the Editor. Correct all errors clearly, and return the galley promptly. Authors will be billed at cost for alterations in the galley proofs involving author's changes from manuscript copy, but not for corrections of printer's errors.

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When a manuscript is accepted by the Editor for publication, the Editor and his staff must prepare the typescript for the printer by proofmarking the submitted copy. In proofmarking, the copy is put in a form consistent with the policy of the journal, but at the same time the individual style and personal preferences of the author are retained as far as possible. The author can do much to assure himself that his published articles will be free from mechanical errors by carefully preparing his typescript, giving special attention to the form used by the journal, especially in regard to tables, figures, and literature citations.

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hyphenated; for example, one fourth, two thirds, two and one half, except in tables and graphs where 1/4, 2/3, 2 1/2 are appropriate. It is best, however, to avoid fractions whenever possible and substitute the decimal form, i.e., 0.5, 0.1 and 2.5.

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